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In vitro co-infection studies on *Toxoplasma gondii* and *Eimeria tenella* in primary poultry macrophages

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For my families

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List of abbreviations

BHK	baby hamster kidney
BMDMs	bone marrow-derived macrophages
CCL	Chemokine ligands
CD	cluster of differentiation
CFT	complement fixiation test
CIT	complement inhibition test
CLSM	confocal laser scanning microscopy
COI	cytochrome *c* oxidase subunit I gene
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle'sMedium
DT	sabin-feldman dye test
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
GFP	green fluorescent protein
Hela	epitheloid cervix carcinoma
HEp-2	human epithelial type 2
HFF	human foreskin fibroblast
HTC	hepatoma tissue culture
IFA	immune fluorescence assay
IFAT	indirect fluorescent antibody test
IFN	interferon
IHAT	indirect haemagglutination test
IL	interleukin
iNOS	nitric oxide synthase
ITS	internal transcribed spacer
LAT	latex agglutination test
LPS	lipopolysaccharide
MAT	modified agglutination test

MDBK	Madin-Darby kidney
MOI	multiplicity of infection
MQ-NCSU	chicken mononuclear cell line (Muquarrab Qureshi-North Carolina State University)
NO	nitric oxide
NTC	non-template control
PAMPs	pathogen associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PV	parasitophorous vacuole
qPCR	quantitative real time PCR
RK-13	rabbit kidney-13
RT	room temperature
SD	standard deviation
Th	T-helper
TLR	toll-like receptor
TNF	tumor necrosis factor
TNLF	tumor necrosis-like factor
Vero	fibroblast-like kidney
YFP	yellow fluorescent protein

1 Introduction and literature review

1.1 Introduction

Both *Toxoplasma (T.) gondii* and *Eimeria (E.) tenella* belong to the phylum Apicomplexa and are widely prevalent in poultry worldwide. Chickens are an important reservoir of *T. gondii*. This is especially true for free-ranging chickens which have been reported to show high seroprevalence rates for *T. gondii* infection (DUBEY 2010). Although toxoplasmosis commonly takes a subclinical course in chickens, it is one of the main foodborne diseases affecting humans by eating uncooked meat or raw fruit and vegetables contaminated with cat feces (JONES and DUBEY 2012). On the contrary, *E. tenella* is host-specific and a parasitic protozoan which invades and develops in the intestinal epithelial cells of chicken causing the economically important eimeriosis (CLARK et al. 2017).

During an infection, immune cells such as macrophages are stimulated to phagocytize the intracellular parasites and produce inflammatory cytokines and chemokines. Chicken macrophages play a crucial role in the host immune response to both *T. gondii* and *E. tenella* (HÉRIVEAU et al. 2000; QUÉRÉ et al. 2013; UNNO et al. 2008). On the other hand, chicken macrophages have been reported as host cells for *T. gondii* tachyzoites reproduction and as transporters of *E. tenella* sporozoites during the endogenous phase of development (CHALLEY and BURNS 1959; MALKWITZ et al. 2013; MEIRELLES and SOUZA 1985), assigning them an ambiguous role in host-pathogen interaction.

Many studies investigate host-pathogen interactions of either *T. gondii* or *Eimeria* spp. infections. However, little is known to date about the mutual pathogen-pathogen interaction between these two parasites and interactive influence on and response from the avian host organism through co-infections are likely to be common in the field. An infection model for *T. gondii* based on *ex vivo* isolated chicken primary monocyte-derived macrophages has been established previously (MALKWITZ et al. 2013). Coccidiosis is considered an excellent model to study the gut immunopathology due to the effectiveness of innate, acquired or maternal immunity against *Eimeria* spp. (CHAPMAN et al. 2013). An early study showed that macrophages isolated from chicken immunized with *E. tenella* inhibited not only the intracellular development of *E. tenella* sporozoites,

but also *T. gondii* tachyzoites (ONAGA et al. 1983). In addition, SOARES MEDEIROS et al. (2011) referred that extraintestinal merozoites of *T. gondii* and intestinal merozoites of *E. tenella* showed a similar functional ultrastructure. Therefore, studies into co-infections with both highly similar pathogens regarding parasite behavior as well as host reaction are needed to clarify their impact on a susceptible avian host.

In the current *in vitro* studies, the co-occurrence of the two pathogens in the same avian immuno-competent host cell population towards potential parasite-parasite as well as altered patterns of parasite-host interactions were investigated. The obtained data will help to understand the relevance of natural co-infections for both, chickens (replication and pathogenicity of *E. tenella*, nature of *T. gondii* pathogenesis in co-infections) and humans, as the replication and tissue colonization by *T. gondii* might be enhanced during mixed infections thus potentially increasing human risk of *Toxoplasma* infection by poultry meat ("One Health").

1.2 Literature review

1.2.1 *Toxoplasma gondii*

1.2.1.1 Toxoplasmosis

T. gondii is the most successful protozoan parasite globally and can infect almost all warm-blooded animals including humans. Chronic toxoplasmosis is estimated to be present in one-third of the global human population (TORGERSON and MASTROIACOVO 2013). Mostly, toxoplasmosis in adults is asymptomatic but it can cause devastating disease in immunosuppressed persons, e.g., systemic lethal infections, ocular disease, congenital disease, and abortion in pregnant women (LYONS et al. 2001; ROBERT-GANGNEUX and DARDÉ 2012). Poultry has a high tolerance and shows rarely clinical signs after infections with *T. gondii* (DUBEY 2010). Several serological tests have been used to detect toxoplasmosis in animals and humans, including the Sabin-Feldman dye test (DT), complement fixation test (CFT), complement inhibition test (CIT), indirect haemagglutination test (IHAT), indirect fluorescent antibody test (IFAT), latex agglutination test (LAT), modified agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA) (DUBEY. 2010). While drugs are available that control acute human toxoplasmosis, such as

pyrimethamine plus sulfadiazine, timely treatment is difficult in subclinical infections. *T. gondii* infects a great range of avian species, including wild birds as well as domestic birds (DUBEY 2002; DUBEY 2010). However, most published studies on parasite-host interaction during *T. gondii* infections and the involvement of macrophages are based on experiments with mammalian macrophages. Little is known about the avian host immune response to *T. gondii*, particularly how this parasite modulates the avian macrophage. In fact, chicken macrophages have been shown to play a crucial role in the innate immune response against chicken toxoplasmosis (GAZZINELLI et al. 1993a). GUILLERMO and DAMATTA (2004a) referred that *T. gondii* is able to inhibit nitric oxide (NO) production not only in murine macrophages but also in a permanent chicken macrophage cell line. MALKWITZ et al. (2018) showed that proinflammatory cytokines such as interleukin (IL)-12 p40 and nitric oxide synthase (iNOS) were upregulated by chicken primary macrophages after *T. gondii* infection.

1.2.1.2 Life cycle

There are three infective stages in the facultative heteroxenous life cycle of *T. gondii*. This includes the sporozoite inside sporulated oocysts, rapidly dividing tachyzoites, and slowly dividing bradyzoites contained in tissue cysts. All three stages are infectious for both intermediate and definitive host by different ways of transmission (TENTER et al. 2000), though oral infection is the most common route. Felines serve as the definitive host in the life cycle *T. gondii* and excrete oocysts that feature a high tenacity in the environment. Sporogony occurs environmentally and leads to the development of infectious oocysts. One oocyst contains two sporocysts, each containing four sporozoites (JACKSON and HUTCHISON 1989). After oral uptake of sporulated oocysts by the susceptible host, sporozoites differentiate into so-called tachyzoites undergoing asexual development and spreading systemically. Tachyzoites actively invade host cells in different tissues and replicate rapidly. Tissue cysts develop as early as 7-10 days after infection as transformation from tachyzoite to bradyzoites (syn. cystozoite) occurs (DUBEY et al. 1998). Cysts are found mainly in brain cells or muscular cells as spheroid structures with a varying size of 10-100 µm. Upon ingestion of cyst-containing tissues by carni- or omnivore intermediate hosts, cysts are ruptured in the digestive tract and bradyzoites are liberated. The released bradyzoites infect the intestinal epithelium and

differentiate back into the tachyozoite stage for multiplication throughout the body of the new host. Tachyzoites can also cross the placenta and infect the fetus (congenital toxoplasmosis) during pregnancy (ROBERT-GANGNEUX and DARDÉ 2012). Sexual reproduction occurs only in the cats' intestine. Here, bradyzoites undergo a limited asexual multiplication to form merozoites during schizogony followed by sexual differentiation to male and female gametes (gametogony). After fertilization, unsporulated oocysts develop within intestinal epithelium and are finally liberated from the host cells to be excreted via the fecal route.

1.2.1.3 Zoonotic importance and prevalence in poultry

TENTER et al. (2000) reported that between 30% and 65% of humans worldwide were infected by *T. gondii*. One of the important transmission routes of *T. gondii* to human is fecal contamination of food and water by infected felids. DUBEY and BEATTIE (1988) reported that the seroprevalence in wild felids may reach up to 100% and average seropositivity is 30-40% in domestic cats. On the other hand, foodborne human toxoplasmosis is commonly acquired by consumption of undercooked cyst-contaminated meat (TENTER et al. 2000). In general, pork, meat originating from ruminants as well as poultry have been considered to be possible sources of *T. gondii* infection in humans (DUBEY 2000). Along with the intensification of livestock management including improved hygiene conditions in housed animals, the risk of *T. gondii* infection in commercial livestock such as pigs and poultry has been reduced significantly (TENTER et al. 2000; DUBEY et al. 2005). Nevertheless, free-ranging livestock is still frequently exposed to *T. gondii* infection, including avian hosts. In poultry, DUBEY (2010) considered that free-range chicken play a crucial role in the transmission of *T. gondii* due to their clinical resistance and their higher life expectancy compared to rodents. Worldwide, high prevalence of *T. gondii* was found in backyard chickens (up to 100%) and free-range organic chickens (30-50%) (DUBEY et al. 2004; KVIČEROVÁ et al. 2008). In Germany, KOETHE et al. (2011) reported recently high seroprevalence of 20.2% in domestic turkeys. High seroprevalence of 5.7% and 25.2%, respectively, were also observed in Germany in ducks and geese (MAKSIMOV et al. 2011).

1.2.1.4 *In vitro* culture system

Several culture systems for *T. gondii* have been described and utilized by many research groups for decades. Mostly, actively dividing tachyzoites are promoted in cell culture systems while reproduction of *T. gondii* oocysts has not been achieved *in vitro* yet (MULLER and HEMPHILL 2013). Parasites and their soluble products have been used for experimental in *vitro* models and the development of serologic tests (ASHBURN et al. 2000; HUGHES and FLECK 1986) or drug screening (FICHERA et al. 1995). The tachyzoite is considered as one of the most infectious stages of the *T. gondii* life cycle (DUBEY 1998). Tachyzoites are able to invade all nucleated cell types where they multiply in a specific parasitophorous vacuole (PV). Due to the infectivity of *T. gondii* to a broad range of susceptible cells, a large variety of suitable mammalian cell lines have been used to investigate the biology of *T. gondii* tachyzoites. This includes HeLa (epitheloid cervix carcinoma), HFF (human foreskin fibroblasts), HEp-2 (human epithelial type 2), Vero (fibroblast-like kidney) cells and kidney cell line (ASHBURN et al. 2000; HUGHES and FLECK 1986; EVANS et al. 1999). Although *T. gondii* tachyzoites are relatively easy to cultivate, the stage conversion into the chronic cyst form containing the bradyzoite stage is more difficult to achieve *in vitro*. It may be induced by abiotic stress factors such as alkaline pH, chemicals, heat shock and some pro-inflammatory cytokines (SKARIAH et al. 2010).

In addition to permanent cell lines, primary cells, such as neural cells, intestinal epithelial cells and primary immune cells, are commonly used to study the biology of *T. gondii* as well as host-parasite interactions (FISCHER et al. 1997; MOURA et al. 2009). In chicken, primary blood monocyte-derived macrophage cultures are suitable host cells for *T. gondii* tachyzoites and allow their reproduction (MALKWITZ et al. 2013; MALKWITZ et al. 2018). Functional studies into the immune response of primary macrophages against *T. gondii* showed elicitation of macrophage-derived pro-inflammatory cytokines (MALKWITZ et al. 2018).

1.2.2 *Eimeria tenella*

1.2.2.1 Clinical coccidiosis in chickens

There is no doubt that coccidia are the most important parasites of poultry regarding distribution,

frequency and economic losses (SHIRLEY and LILLEHOJ 2012). Seven species of host-specific *Eimeria* are commonly found in chickens, namely *E. acervulina*, *E. mitis*, *E. maxima*, *E. brunetti*, *E. necatrix*, *E. praecox*, and *E. tenella*. *E. tenella* is particularly considered as a rather pathogenic species in chicken. This species causes hemorrhagic intestinal damage as well as economic and animal losses in poultry industry and is of relevance to animal welfare (DALLOUL and LILLEHOJ 2006; SHIVARAMAIAH et al. 2014). Secondary infections with bacterial as well as viral pathogens are commonly observed to mutually interact with parasites thus promoting more serious clinical signs (RUFF. 1999).

Diagnosis of *E. tenella* infection is based on typical clinical signs, characteristic gross lesions in caeca upon necropsy, and oocyst identification in faecal droppings by microscopic analysis (CHAPMAN et al. 2013). Polymerase chain reaction (PCR) assay is commonly used to detect and differentiate *Eimeria* species (KAWAHARA et al. 2008). Intragenomic polymorphisms of the 18S rRNA gene or internal transcribed spacer (ITS) regions (BLAKE et al. 2008; OGEDENGBE et al. 2011b) have been used to characterize species and strains of *Eimeria*. The mitochondrial cytochrome *c* oxidase subunit I gene (*cox-1*, COI) represents another good target for molecular identification by PCR (OGEDENGBE et al. 2011a).

1.2.2.2 Life cycle

Unlike *T. gondii*, chickens are the only and specific host over the entire life cycle of *E. tenella* (BLAKE and TOMLEY 2014). The life cycle contains exogenous and endogenous developmental stages. The unsporulated oocyst (non-infective) is excreted with the faeces by infected chickens, and sporulates into the infective oocyst in the environment based on the availability of appropriate (warm) temperature, moisture, and oxygen. One sporulated oocyst contains four sporocysts, each containing two sporozoites. When sporozoites are released from sporulated oocysts after oral uptake by the next susceptible chicken host, *E. tenella* sporozoites invade enterocytes undergoing endogenous development in the caecal mucosa (LAL et al. 2009). At first, trophozoites are formed from sporozoites within the host cells. Subsequently, merozoites are produced during so-called schizogony (merogony), which are released under host cell destruction and then penetrate into neighbouring enteric cells. After completion of another schizogony, second generation merozoites are released and

differentiate after invasion of mucosal host cells into either microgamonts or macrogamonts, a process of sexual reproduction called gametogony (syn. gamogony). Finally motile microgametes leave their host cell seeking out for macrogametes to form the diploid zygote (syngamy) that transforms into an unsporulated oocyst by developing a firm oocyst wall out of so-called wall forming bodies. The whole endogenous part of the life cycle of *E. tenella* is short with only 5 days of prepatency that may lead to rapid environmental contamination with large numbers of oocysts that become infective following meiosis to form haploid sporozoites within a short period of time (JEURISSEN et al. 1996).

1.2.2.3 Control

The poultry industry is one of the most important food suppliers all around the world. Coccidiosis costs more than $3 billion dollars annually in the global poultry industry due to production losses and cost of prevention and treatment (DALLOUL and LILLEHOJ 2006). *Eimeria* spp. transmission occurs easily by feed, water, oocyst-contaminated litter and persons moving between houses and farms (BELLI et al. 2006). Commercial flocks (broilers) are generally kept in an environment (temperature and humidity) which promotes exogenous *Eimeria* survival and sporulation. Control of coccidiosis is essential to avoid significant economic losses.

Control of coccidiosis in poultry is currently achieved by appropriate management, use of drugs or vaccination. Prophylactic anticoccidials such as polyether ionophores or synthetic products were used effectively over decades in the poultry industry. Ionophores are able to destroy motile sporozoites and merozoites of *Eimeria* in the gut lumen or following cell penetration (SMITH and STROUT 1979). In the United States, anticoccidials were commonly used in about 99% of commercial broiler chicken farms from 1995 to 1999 (CHAPMAN 2001). However, drugs are no longer completely effective on many operations as resistance to anticoccidial drugs has developed in *Eimeria* spp. (RATHINAM and CHAPMAN 2009; PEEK and LANDMAN 2006). Some natural herbs have been found effective as alternative treatment against *E. tenella* by reducing lesion scores and oocyst excretion (YOUN and NOH 2001; ALLEN et al. 1997). Live attenuated or non-attenuated vaccines have been well established in recent years. However, the cost of vaccine production and acquisition of suitable parasite strains have been barriers to the general use (CHAPMAN and

JEFFERS 2014).

1.2.1.4 *In vitro* culture system

PATTON (1965) firstly reported a method for *in vitro* culture of *E. tenella* in Madin-Darby bovine kidney (MDBK) cells. TIERNEY and MULCAHY (2003) tested *in vitro* reproduction efficiency for the *E. tenella* asexual stage in a range of cell lines, showing that parasites displayed significant replication in BHK (baby hamster kidney), MDBK and RK-13 (rabbit kidney) cells. MDBK cells and an incubation temperature of 41 ℃ were considered optimal for the *in vitro* growth of *E. tenella*.

In vitro cultivation of *E. tenella* has been applied to test for anticoccidial efficacy of synthetic and natural compounds as alternative to animal experiments (KHALAFALLA et al. 2011; THABET et al. 2017; ALNASSAN et al. 2015). The molecular and immunologic features of the parasite have also been investigated *in vitro* (LILLEHOJ and CHOI 1998). For instance, transient transfection of *E. tenella* oocysts has been established *in vitro* and assists the interpretation of genetic, biologic and morphologic studies (SHI et al. 2008; CLARK et al. 2008). Although there were some studies describing propagation of *E. tenella* over the whole life cycle in primary chicken kidney cells, pathogenicity was reduced by generation (ZHANG et al. 1997; HOFMANN and RAETHER 1990; SHI et al. 2008). Reliable protocols for efficient *in vitro* development to the sexual stages are still not available.

Chicken macrophages have been broadly used to study parasite biology and the innate immune response to *E. tenella* (LONG and ROSE 1976). It was shown that primary cultures of chicken macrophages from peritoneal exudates allowed only limited growth of *E. tenella*. Macrophages isolated from spleen were used to study *E. tenella* infection-related cytokine expression previously (BYRNES et al. 1993). In addition, chicken macrophage permanent cell lines such as macrophage-like chicken (HD-11) and hepatoma tissue culture (HTC) cell lines reacted similarly to primary macrophages to *E. tenella* (ZHANG et al. 1995a; CHOW et al. 2011).

1.2.3 Macrophage-parasite interaction

1.2.3.1 General aspects

Chicken have evolved a sophisticated immune system that differs from that of mammals in various aspects. As in mammals, chicken macrophages constitute the first line of immunological defense against pathogens (QURESHI et al. 2000). They act as antigen presenting cells for B and T lymphocytes. Their functions are primarily phagocytosis and production of chemokines and cytokines that mediate both humoral and cell-mediated inflammatory responses (DALLOUL and LILLEHOJ 2006). Nutrition, genetics and environmental factors are thought to modulate macrophage functions (QURESHI 2003). Unlike in the murine immune system, only few resident macrophages are present in the abdominal cavity which is thus not very suited as a source of chicken macrophages. In chicken, harvesting of blood monocytes is a feasible procedure to gain adherent macrophages for *in vitro* studies. Avian monocyte-derived macrophages constitute a major phagocytic cellular component in the blood. Primary macrophage cultures have been established by *in vitro* cultivation of peripheral blood white cells and rinsing off non-adherent cell fractions (QURESHI et al. 2000). Additionally, several chicken macrophage-like cell lines have been developed with more consistent functional characteristics than primary cultures, such as HD 11, chicken mononuclear (Muquarrab Qureshi-North Carolina State University, MQ-NCSU) cell line as well as HTC cells (BEUG et al. 1979; QURESHI et al. 1990; INOUE et al. 1992; XING et al. 2010).

NO is an important immunologic mediator produced by iNOS, that is involved in antimicrobial activity against intracellular pathogens (KODUKULA et al. 1999; MURRAY and NATHAN 1999). iNOS is considered to be mainly secreted by activated macrophages (HIBBS et al. 1987). Avian macrophages can be stimulated e.g. by lipopolysaccharide (LPS) contact or inflammatory cytokines. Similar to mammalian macrophage activation, LPS-stimulated chicken macrophages exhibit activation of iNOS as well as tumor necrosis factor (TNF) (QURESHI et al. 1993). Moreover, activation of chicken macrophages by interferon (IFN)-γ plays a crucial role in the Th1 mediated protection (HE et al. 2011). In addition, Toll-like receptors (TLRs), usually expressed by macrophages, play crucial roles in the phagocytosis and activation of innate immunity (YILMAZ et al. 2005). TLRs are able to recognize a variety of pathogen associated molecular patterns (PAMPs) in microbes, such as bacterial LPS, lipopeptids, glycolipids and viral nucleic acids (LEMAITRE 2004).

1.2.3.2 Phagocytosis

Macrophages protect the host by engulfing microbes or apoptotic cells within phagosomes where they are rapidly destructed. The non-specific function, phagocytosis, appears as early as day 12 of chicken embryonic development (JEURISSEN and JANSE. 1989). Macrophages can destroy both intracellular and extracellular pathogens through phagocytosis and lysosomal degradation. Cultured primary macrophages and macrophage cell lines can spontaneously phagocyte e.g. various bacterial or fungal pathogens, and phagocytosis may be employed even to visualize macrophage activity by uptake of fluorescent microbeads (OKAMURA et al. 2005; ROSSI and TURBA 1981; ABEL et al. 1991). Most of the phagocytosis is mediated by specific receptors such as mannose receptors on the surfaces of macrophages. These specific receptors can recognize antigenic targets of certain pathogens and bind pathogens to the macrophages for phagocytosis (QURESHI 2003). After pathogens are phagocytized and transferred into the phagosome of macrophages, the fusion of the phagosome with a lysosome occurs which leads to the formation of the phagolysosome aiming for degradation of the internalized pathogen (QURESHI and DIETERT 1995; AMER and SWANSON 2002). In avian macrophages lysosomes contain anti-microbial proteins and enzymes such as acid phosphatase and β-glucuronidase (SHARMA 2018; JUUL-MADSEN et al. 2008).

T. gondii actively penetrates into both phagocytic and non-phagocytic cells and resides in a non-fusogenic vacuole that excludes most host-derived proteins thus protecting the pathogen from host cell endocytic and exocytic pathways (MORDUE et al. 1999; SIBLEY 2011). The host cell membrane contributes to more than 85% of the PV membrane. In fact, formation of *T. gondii* PV is based on invagination of the host cell plasma membrane within 25-40 s after the beginning of host cell invasion (MORISAKI et al. 1995; LING et al. 2006; ZHAO et al. 2009). The *T. gondii* PV is a target for recognition by the host immune system. MEIRELLES and SOUZA (1985) reported that chicken monocyte-derived macrophages were capable of fusing the PV formed by amastigote *Leishmania mexicana* with lysosomes after infection. However, *T. gondii* tachyzoites multiply well in macrophages derived from chicken blood monocytes within specific PV. *T. gondii* PVs were shown to fail triggering of phagosome acidification in macrophages (SIBLEY et al. 1985). Murine phagocytic leukocytes such as monocytes, lymphocytes and neutrophils are able to disseminate *T.*

gondii tachyzoites from the intestine to the brain (COURRET et al. 2006). LING et al. (2006) and ZHAO et al. (2009) demonstrated that murine macrophages are involved in the vacuolar disruption of *T. gondii* PV. CD 40-activated human macrophages were capable to destruct intracellular *T. gondii* by triggering autophagy-dependent fusion of PV and lysosomes (ANDRADE et al. 2006). Moreover, macrophage phagocytosis plays an important role in triggering the cellular immune response against intracellular *T. gondii*. For instance, human monocyte-derived macrophages failed to produce cytokines in response to actively invading *T. gondii* when macrophage phagocytosis was inhibited by mycalolide B (TOSH et al. 2016).

For *Eimeria*-infected animals, macrophages have been shown to phagocytize the parasite and may even serve as vehicles for transporting sporozoites into the lamina propria of the intestinal mucosa (TROUT and LILLEHOI 1993; CHALLEY and BURNS 1959). In an early *in vitro* study, LONG (1975) observed intracellular *E. tenella* sporozoites within 2 hours after infection and very poor parasite growth occurred in cultured chicken macrophages. VERVELDE et al. (1996) reported that sporozoites were frequently located within or next to the lamina propria infiltrated with macrophages after *E. tenella* infection in naïve chickens. ROSE and LEE (1977) indicated that the mechanism of sporozoites entry into macrophages was by phagocytosis without any evidence of active penetration. Similarly, TAUBERT et al. (2009) referred that the active invasion of *E. bovis* sporozoites was negligible during *in vitro* infection in bovine macrophages.

1.2.3.3 Chicken macrophages in innate immunity

1.2.3.3.1 Innate immune response against *T. gondii*

As in humans and mammals, infection of chicken monocytes by *T. gondii* tachyzoites triggers innate immune responses e.g. by stimulation of synthesis of pro-inflammatory cytokines, followed by activation of adaptive immune responses. Intracellular *T. gondii* can be killed by human monocyte-derived macrophages activated by IFN-γ belonging to the cluster of differentiation (CD) 40 (ANDRADE et al. 2006). Macrophage activation plays a crucial role in mucosal immunity against *T. gondii* by inducing T-helper (Th)1 immune responses, including iNOS upregulation, IL-12 production and TNF-α secretion (DUNAY et al. 2008). Moreover, IFN-γ controls the replication of

this intracellular parasite by activation of macrophages. GAZZINELLI et al. (1993b) showed that down-regulation of IFN-γ and TNF-α reduced macrophage activation, leading to parasite growth as well as tissue damage. Human peripheral blood monocytes have been shown previously to be important sources of IL-12 and TNF-α in host defense against *T. gondii* (TOSH et al. 2016). Macrophages activated by *T. gondii in vitro* produce IL-1β and TNF-α (PHILIP and EPSTEIN 1986; NAGINENI et al. 1996). In addition, macrophages are one of the best characterized iNOS expression sources. *T. gondii* Type I and Type II strains are affected by iNOS activity in murine cells *in vivo* (TAKÁCS et al. 2012). SCHARTON-KERSTEN et al. (1997) showed that mortality of iNOS knockout mice infected with *T. gondii* was high at the stage of parasite expansion. Additionally, IL-10 is important factor in *T. gondii* infection in mice, which is involved in down-regulating inflammatory response (GAZZINELLI et al. 1996).

1.2.3.3.2 Innate immune response against *E. tenella*

LONG and PIERCE (1963) referred that B cells and the humoral immunity played a minor role during primary infection of chickens with *E. tenella*, implying the importance of cell-mediated responses against this parasite. The host immune responses to *E. tenella* are complex and depend on the stage of parasite development, nutritional condition of infected chicken as well as the prior host exposure in terms of acquired immunity (LILLEHOJ et al. 2004). VERVELDE et al. (1996) reported that most infiltrating leukocytes were macrophages and T cells in the lamina propria of *E. tenella*-infected intestine in chickens. *In vitro E. tenella* development was inhibited by IFN-γ activated chicken macrophages (DIMIER et al. 1998). Tumor necrosis-like factor (TNLF) is considered to play a major role in weight loss in *E. tenella*-infected chickens (ZHANG et al. 1995b). In the *in vivo* studies of DALLOUL et al. (2007) and LAURENT et al. (2001), iNOS expression was associated to hemorrhagic enteritis after infection. The presence of significant serum NO was associated with the reduction of faecal oocyst numbers (ALLEN 1999). *In vitro* replication of *E. tenella* was inhibited by macrophages previously exposed to NOS stimulation (DIMIER-POISSON et al. 1999). Altogether, macrophages are key immunocytes of the host innate immune response and produce cytokines to control *E. tenella* replication/reproduction thus reducing pathologic severity of the disease in chicken.

Macrophages were activated and elicited various chemokines and cytokines after infection of previously naïve chicken (LILLEHOJ 1998). In *Eimeria* infection, many cytokines are not only involved in mediating Th1-type responses during the early stage of infection but also some Th2 response. IL-1 expression by macrophages was observed following *in vitro* infection (BYRNES et al. 1993). Chicken macrophages were main sources of production of TNF-like factor during coccidial infection (ZHANG et al. 1995a). Cytokines such as IL-1β, IL-6 and IL18 were upregulated after *Eimeria* infection. In addition, chemokine ligands (CCL) and chemokine family members such as CCLil, CCLi3 and CCLi7, were induced by infection (DALLOUL et al. 2007). A range of chemokines and cytokines were produced differently following primary and secondary infection with *Eimeria* (LILLEHOJ et al. 2003). ZHANG et al. (1995a) reported that expression of TNLF was observed in peripheral blood macrophages after primary, but not secondary infection by *E. tenella*. Several studies have been conducted to understand the interaction between *E. tenella* infection and the chicken immune response. However, the roles of parasite co-working with other pathogens such as *T. gondii* in mediating chicken innate immune responses by macrophages remain yet to be understood.

1.2.4 Co-infection

1.2.4.1 Natural and experimental co-infection with parasites

Concomitant infections between protozoan parasites and other pathogens, parasitic or other, are common in natural infections in animals and humans. For example, natural co-infections with *Plasmodium* species (malaria) and different helminths are often seen in humans (BROOKER et al. 2007; FELGER et al. 1999). A case report on a 6-week-old dog described the concurrent presence of six different parasite species including tick-borne and intestinal parasites (GAL et al. 2007). In chickens, there were descriptions of concurrent natural infections with *Cryptosporidium* species and a variety of chicken viruses, including reovirus (RITTER et al. 1986), Marek's disease virus (FLETCHER et al. 1975) and chicken anaemia virus (DOBOS‐KOVÁCS et al. 1994). Co-occurrence of *T. gondii* and *Eimeria. spp* in the same hosts has been also reported previously. For example, MANWELL et al. (1945) found that sparrows were suffering from heavy infections (blood and organs) by *T. gondii* and *Eimeria. spp*. A seroprevalence report on wild rabbits in Scotland

showed co-infection with *T. gondii* and *Eimeria stiedai* (MASON et al. 2015).

Although most parasitological studies focus on the features of single parasite species and host-single parasite interactions, host-parasite interaction in mixed infections as well as parasite interaction with other pathogens is more and more considered important to explain pathogenesis, disease outcome and to design procedures for pathogen management (TELFER et al. 2008). For instance, some protozoa can cause severe disease or even lethal outcomes in patients or animals immunosuppressed due to the presence of other infectious agents. BROOKER et al. (2007) reported that co-infections with malaria and hookworms gave rise to higher human anemia burden in Africa. Chickens co-infected with *Cryptosporidium* and reovirus appeared more depressed and excreted higher numbers of both agents (GUY et al. 1988).

Moreover, cross-effective immune response occurs between pathogens with antigenically homologous genes or polarized immune response, leading to modification of the host immune protection. YOELI et al. (1955) and JAHIEL et al. (1968) investigated that virus-induced IFN-α might have a protective effect in humans against malaria parasites. NIIKURA et al. (2008) demonstrated that infections with nonlethal *Plasmodium* species suppressed weight loss and the severity of disease caused by parasitemia of highly pathogenic species and reduced liver injury. On the other hand, inappropriate regulation of phagocytosis and proinflammatory cytokine production were found in macrophages due to HIV infection may impair control of malaria in humans (LUDLOW et al. 2012).

Parasites may also directly interact with pathogens which infect the same site within a host leading to antagonistic interplay or co-localization. FRONTERA et al. (2005) reported an experimental co-infection with the intestinal worm *Ascaris suum* and the lungworm *Metastrongylus apri* in pigs, and suggested competition between these two parasites.

1.2.4.2 Co-infection studies on *T. gondii*

Parasite reproduction as well as host morbidity and clinical aspects during toxoplasmosis can be affected by co-infections with other parasites. Host immune responses are altered during co-infection by *T. gondii* and other parasites, including *Schistosoma mansoni* and *E. acervulina* (ARAUJO et al.

2001; HIOB et al. 2017). GUERRERO et al. (1997) reported previously that *T. gondii* multiplication was enhanced during co-infection with *Trypanosoma lewisi* in white rats. In contrast, *T. gondii* is capable to support the replication of malaria parasites in rats (RIFAAT et al. 1984). Mutual influences occur not only with Th1 response stimulating protozoa, but also with helminths that generally induce a Th2 response. MARSHALL et al. (1999) proved that mice displayed more weight loss and mortality during dual infection by *T. gondii* and the blood fluke *S. mansoni*. Furthermore, ARAUJO et al. (2001) observed that liver acutely inflamed due to *T. gondii* was associated with high levels of pro-inflammatory cytokines such as TNF-α and IL-12 in co-infection with *S. mansoni*. Investigations into *T. gondii* co-infections with intracellular pathogens showed it co-existed with *Leishmania infantum* in the same single host cell (CHRISTODOULOU et al. 2011). However, *T. gondii* dominated over bacterial *Chlamydiae* in dually infected cells (ROMANO et al. 2013).

1.2.4.3 Co-infection studies on *Eimeria. spp*

Mutual interaction with other microbes has also been studied for *Eimeria* species. Both mortality and severity of lesions increased in experimentally *E. tenella* and *Clostridium perfringens* co-infected chicken embryos compared to mono-infected embryos (ALNASSAN et al. 2013). *Lactobacillus* species isolates have been shown to significantly inhibit *E. tenella in vitro* invasion in MDBK cells (TIERNEY et al. 2004). In mixed *Eimeria* species infections, it seems that each *Eimeria* species can co-localize well with others in the gut of chickens (CORNELISSEN et al. 2009). Although host immune response is altered during the mixed infection of different *Eimeria* spp., individual *Eimeria* species-specific intestinal lesions showed no obvious difference compared to single species infections (CORNELISSEN et al. 2009). In particular, there is no competitive effect in mixed *Eimeria* species infections. However, JENKINS et al. (2008) demonstrated that clinical signs of coccidiosis caused by *E. maxima* were reduced in chickens also infected with *E. praecox*, but oocyst excretion was not affected for both parasites. They also reported that *E. praecox* did not appear to induce protective immunity against *E. maxima* during co-infection (JENKINS et al. 2009).

2 Overview of own scientific work

2.1 Aims

Complex interactions between parasites and host may arise during co-infection with two pathogens such that the burden of one or both of the infectious agents may be increased, one or both may be suppressed, or one may be increased and the other suppressed. *In vitro* cell culture of protozoa in chicken primary macrophages allows simultaneous as well as sequential infection of the same host cell culture by parasites, in this case *T. gondii* and *E. tenella*. The following hypotheses summarize the aim of this work:

1. Chicken primary monocyte derived macrophages are suitable as a tool for *in vitro* co-infection with both *T. gondii* tachyzoites and *E. tenella* sporozoites.

In order to test and visualize the parasite development of *T. gondii* tachyzoites and *E. tenella* sporozoites, a model is required to cover the intended period of co-infection by both parasite species. Therefore, parasite infection doses were optimized for establishing a 3-day infection cultivation of chicken macrophages, a period supposed to be suitable to keep primary macrophages alive and functional. Virulent parasites such as *T. gondii* RH strain and *E. tenella* Houghton strain were used to infect chicken macrophage cultures. To evaluate parasite growth and cell morphology alteration conventional light microscopy and confocal laser scanning microscopy (CLSM) were applied (Publications 1, 2, 5 and Manuscript 3, 4). In addition, parasite growth and relation between parasites and phagocytic cells during mono- or co-infection were observed within 24 hours post infection (Manuscript 3).

2. Parasite development of both *T. gondii* and *E. tenella* is mutually modulated in co-infected cell cultures.

In order to understand potential interactions between both taxonomically related parasites during simultaneous or sequential co-infection, assessment of *T. gondii* and *E. tenella* replication was performed in comparison to mono-infections with either parasite. Long-term infection was carried out to evaluate the capacity of parasite reproduction over 72 hours in chicken primary monocyte

derived macrophages by quantitative real time PCR (qPCR) (Publication 2). Likewise, quantities of intracellular parasites were estimated during the simultaneous or sequential (*E. tenella* followed by *T. gondii* infection) co-infection to evaluate the extent of parasite invasion and phagocytosis by macrophages at the early phase of infection (24 hours) under the variable co-infection conditions (Manuscript 3 and 4).

3. Phagocytosis capacity and regulation of cytokines by chicken primary macrophages are altered by *T. gondii* and *E. tenella* co-infections.

In order to understand potential host cell reaction during simultaneous co-infection with *T. gondii* and *E. tenella* compared to mono-infections, innate immunity and phagocytosis capacity of chicken primary monocyte derived macrophages were evaluated. Key cytokines were selected and their expression was quantified by qPCR to understand macrophage innate response to both parasites (Publication 2). To study host cell phagocytosis, macrophages were activated by specific pH-sensitive fluorescent bioparticles before or after infection and their capacity to engulf bioparticles was visualized by CLSM over 24 hours (Manuscript 3).

4. Invasion/phagocytosis patterns of *T. gondii* in macrophage cultures are affected by co-infection with *E. tenella* in the early phase of parasite invasion.

In order to understand the dynamic interaction between host cells and parasites, fluorescent *T. gondii* RH-GFP tachyzoites were monitored in *E. tenella* co-infected and *T. gondii* mono-infected cell cultures over 6 hours by CLSM. Parasite tracking was performed for *T. gondii* to assess the mobility of tachyzoites before and after host cell invasion. The survival of *T. gondii* was also recorded by a dye staining of dead cells and parasites during the cultivation (Manuscript 4).

2.2 Publications

2.2.1 Publication 1: Irene Malkwitz, Angela Berndt, **Runhui Zhang**, Arwid Daugschies, Berit Bangoura. Replication of *Toxoplasma gondii* in chicken erythrocytes and thrombocytes compared to macrophages. Parasitology Research. 2017; 116:123-131.

2.2.2 Publication 2: **Runhui Zhang**, Ahmed Thabet, Lysanne Hiob, Wanpeng, Zheng, Arwid Daugschies, Berit Bangoura. Mutual interactions of the apicomplexan parasites *Toxoplasma gondii* and *Eimeria tenella* with cultured poultry macrophages. Parasite & Vectors. 2018; 11:453.

2.2.3 Manuscript 3: **Runhui Zhang**, Wanpeng Zheng, Arwid Daugschies, Berit Bangoura. Apicomplexan co-infections impair with phagocytotic activity in avian macrophages. Submitted to BMC Veterinary Research, 01.2020

2.2.3 Manuscript 4: **Runhui Zhang**, Wanpeng Zheng, Arwid Daugschies, Berit Bangoura. Monocyte-derived chicken macrophages exposed to *Eimeria tenella* sporozoites display reduced susceptibility to invasion by *Toxoplasma gondii* tachyzoites. Submitted to Frontiers in Cellular and Infection Microbiology, 02.2020.

2.2.4 Publication 5: A modified method for purification of *Eimeria tenella* sporozoites. Zaida Renter ́a-Sol ́s and **Runhui Zhang**, Shahinaz Taha, Arwid Daugschies. Parasitology Research. 2020; doi.10.1007/s00436-020-06602-w

2.2.1 Publication 1: Replication of *Toxoplasma gondii* in chicken erythrocytes and thrombocytes compared to macrophages.

Irene Malkwitz, Angela Berndt, **Runhui Zhang**, Arwid Daugschies, Berit Bangoura

Published in Parasitology Research. 2017; 116:123-131

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DOI: https://doi.org/10.1007/s00436-016-5268-y

Author‘s contribution:

I. Investigation

Runhui Zhang was responsible for performing cell imaging.

II. Analysis

Runhui Zhang was responsible for CLSM image creation and analysis of results

2.2.2 Publication 2: Mutual interactions of the apicomplexan parasites *Toxoplasma gondii* and *Eimeria tenella* with cultured poultry macrophages.

Runhui Zhang, Ahmed Thabet, Lysanne Hiob, Wanpeng, Zheng, Arwid Daugschies, Berit Bangoura

Published in Parasite & Vectors. 2018; 11:453

Author‘s contribution:

I. Concept

Runhui Zhang was responsible for the idea and design of this work with the critical evolution and inspection of research goal and aims by Berit Bangoura and Arwid Daugschies.

II. Investigation

Runhui Zhang conducted the research and investigation process including performing the experiments and data collection with the partial participation of Ahmed Thabet (parasite sample preparation and experiment improvement)

III. Analysis

Runhui Zhang performed data analysis with the partial participation of Wanpeng Zheng (CLSM imaging analysis) and Lysanne Hiob (improvement of cytokine analysis).

IV. Manuscript

Runhui Zhang wrote the initial draft and prepared the published work with critical revision and writing improvement by Ahmed Thabet, Lysanne Hiob, Wanpeng Zheng, Berit Bangoura and Arwid Daugschies.

Zhang *et al. Parasites & Vectors* (2018) 11:453
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Parasites & Vectors

RESEARCH Open Access

Mutual interactions of the apicomplexan parasites *Toxoplasma gondii* and *Eimeria tenella* with cultured poultry macrophages

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Abstract

Background: *Toxoplasma gondii* and *Eimeria tenella* are two common parasites in poultry. Mixed infections are likely to occur frequently in chickens due to the high prevalence of both pathogens. In this study, we investigate the co-occurrence of the two pathogens in the same immunocompetent host cell population towards potential parasite-parasite as well as altered patterns of parasite-host interactions.

Methods: Primary macrophages from chicken blood were co-infected *in vitro* with *T. gondii* tachyzoites (RH strain) and *E. tenella* sporozoites (Houghton strain) for 72 h. Morphological observations by light microscopy and assessments of parasite replication by quantitative real-time PCR (qPCR) were performed at 24, 48 and 72 h post-infection (hpi). Six host cell immune factors previously linked to *T. gondii* or *E. tenella* infection were selected for gene expression analysis in this study.

Results: Distinct morphological changes of macrophages were observed during mixed infection at 24 hpi and immunological activation of host cells was obvious. Macrophage mRNA expression for iNOS at 48 hpi and for TNF-α at 72 hpi were significantly elevated after mixed infection. Distinct upregulation of IL-10 was also present during co-infection compared to *Eimeria* mono-infection at 48 and 72 hpi. At 72 hpi, the total number of macrophages as well as the number of both parasites decreased markedly. As measured by qPCR, *E. tenella* population tended to increase during *T. gondii* co-infection, while *T. gondii* replication was not distinctly altered.

Conclusions: Mutual interactions of *T. gondii* and *E. tenella* were observed in the selected co-infection model. The interactions are supposed to be indirect considering the observed changes in host cell metabolism. This study would thus help understanding the course of co-infection in chickens that may be relevant in terms of veterinary and zoonotic considerations.

Keywords: *Toxoplasma gondii*, *Eimeria tenella*, Co-infection, *in vitro*, Chicken macrophage

Background

Toxoplasma gondii and *Eimeria tenella* are two coccidian parasites in poultry. *Toxoplasma gondii* is a heteroxenous parasite and may inhabit a wide range of vertebrate species as intermediate host, which may harbor the cyst stages in various tissues [1] following tachyzoite replication. Several reports showed high seroprevalence of *T. gondii* in chickens worldwide [2–4]. In contrast, *E. tenella* is very host-specific and monoxenous, infecting particularly the caecal mucosa of chicken [5, 6].

Mixed infections are likely to occur in chickens, particularly under free-ranging conditions. Many studies investigate host-pathogen interactions of single *T. gondii* or *Eimeria* spp. infections [7–9], but little is known to date about the pathogen-pathogen-host cell interactions for simultaneous co-infections with species of these two parasite genera. Tachyzoites of *T. gondii* and merozoites of *Eimeria* differ in many features; however, asexual stages of these parasites share ultrastructural similarities [10, 11]. It was shown that transgenic *E. tenella* (Et-TgSAG1) may induce certain immunoprotection against *T. gondii* [12]. On the

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other hand, *T. gondii* as a vaccine vector has a partially protective effect against coccidiosis [13]. Mixed infections with *T. gondii* and *Eimeria* spp. were found in wild rabbits in a recent case report [14]. The ability of *T. gondii* to suppress the macrophage-associated defense to *Mycobacterium avium* has been shown *in vitro* [15].

Chicken macrophages are crucially involved in the host immune response to both *T. gondii* and *E. tenella* [2, 16, 17]. In coccidiosis, chicken macrophages are also involved in sporozoite transport during the endogenous phase of *Eimeria* development [18]. Thus, it appears likely that simultaneous appearance of these two apicomplexan parasites may affect the reaction of host macrophages to parasite infection. Experimental *in vivo* studies revealed a partial mutual interaction between *T. gondii* and *E. tenella* [19] in chickens.

Immune response of primary monocyte-derived macrophages is stimulated *in vitro* by *T. gondii* tachyzoites [20] and *E. tenella* sporozoites [21]. High expression of pro-inflammatory Th1 cytokines is typically related to macrophage function in *T. gondii* infection [22]. Previous investigations also showed that Th1/Th2 pro-inflammatory cytokines related to macrophages are involved in the host response to *Eimeria* infection [23–25]. High production of Th2 inflammatory mediators such as interleukin 6 (IL-6) was reported in *T. gondii* and *E. tenella* mono-infections [26, 27]. *In vitro* replication of *T. gondii* is enhanced significantly when murine macrophages are pre-treated with IL-6 prior to infection [28]. *In vitro* infection of avian macrophages by *E. tenella* sporozoites upregulates nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) transcription [29, 30]. Induced NO production by macrophages is generally related to cytokines such as IFN-γ and tumor necrosis factor α (TNF-α) which play a vital role in immunity of chicken against coccidiosis and toxoplasmosis [31, 32]. Various effects of *in vivo* co-infection of *T. gondii* and *E. tenella* in chickens [19] were observed on IFN-γ, TNF-α, IL-10 and IL-12.

In this study, we aimed to understand the simultaneous co-occurrence of the two pathogens in the same avian immune-competent host cell population and the interaction with the innate immunity against each single pathogen. Additionally, pathogen-pathogen interactions in terms of invasion and replication potential were investigated. The ability of macrophages to host both parasites simultaneously is also demonstrated.

Methods

Macrophages and parasites

Macrophages were separated and collected from chicken peripheral blood mononuclear cells (PBMC) according to established protocols [33]. The isolated PBMC (5×10^6 cells per well) were suspended in RPMI-1640 medium (Sigma, Taufkirchen, Germany) supplemented with 5% chicken serum and 5% fetal bovine serum, penicillin (100 U/ml, PAA), streptomycin (0.1 mg/ml, PAA), and amphotericin B (0.0025 mg/ml, PAA), seeded into 24-well plates and incubated at 41 °C with 5 % CO_2. They were grown to about 90% confluence within 96 h cultivation time. Free transgenic *T. gondii* RH-GFP tachyzoites (type I strain, kindly provided by Professor Dominique Soldati-Favre, University of Geneva Medical School, Switzerland) were harvested from infected human foreskin fibroblast (HFF) cultures by mechanical destruction. *Eimeria. tenella* Houghton strain (kindly provided by Professor Damer Blake, Royal Veterinary College, UK) and transgenic Houghton-YFP strain (kindly provided by Professor Xun Suo, China Agricultural University, China) sporozoites were gained by oocyst excystation following an established protocol [34]. Briefly, the oocyst wall of *E. tenella* was destroyed mechanically with 0.5 mm glass beads (BioSpec Products, Bartlesville, OK, USA). Excystation of sporozoites was performed by incubation with 0.25% trypsin (w/v) (Carl Roth, Karlsruhe, Germany) and 4% sodium taurocholic acid (w/v) (Sigma-Aldrich, Taufkirchen, Germany) at 41 °C for 90 min. Purified sporozoites were collected by passage through columns of nylon wool and DE-52 resin (Whatman, GE Healthcare, USA) with 1% glucose phosphate-buffered saline (PBS) at pH 7.6 (follow buffer). *E. tenella* Houghton strain sporozoites were used for all experiments except for laser scanning analyses where *E. tenella* Houghton-YFP strain was utilized.

Mono- and co-infections

Infection doses for both parasites were optimized prior to the co-infection trial (data not shown). Six groups were set-up for *in vitro* infection studies in primary macrophages: TH, *T. gondii* infection with 5×10^5 tachyzoites (high-dose); TL, *T. gondii* infection with 2.5×10^5 tachyzoites (low-dose); EH, *E. tenella* infection with 5×10^5 sporozoites (high-dose); EL, *E. tenella* infection with 2.5×10^5 sporozoites (low-dose); CI (co-infection), mixed infection with *T. gondii* 2.5×10^5 tachyzoites and *E. tenella* 2.5×10^5 sporozoites; NC, uninfected negative control cell cultures. At 96 h after isolation of PBMCs, purified primary macrophages were infected with parasites according to their group and incubated at 41 °C. At 12 hours post-infection (hpi), the wells were rinsed once with PBS to remove extracellular sporozoites. The medium was changed and cell cultures were further incubated at 41 °C. The course of infection was monitored until 72 hpi.

Positive control MDBK culture infection

In addition to the primary macrophages, Madin-Darby Bovine Kidney (MDBK) cell line cultures were used. In those cell cultures, six infection groups were formed

(TH, TL, EH, EL, CI and NC) as described before. They were carried along in parallel to macrophage trials as controls for parasite replication analysis without macrophage-specific influence on parasite-parasite interaction. MDBK cultures were maintained and infected at 41 °C to enable comparison to primary chicken macrophage cultures under comparable incubation conditions. MDBK host cell cultures were tested before to be easily maintained and multiplied at this temperature (data not shown).

Assessments of parasite replication

Parasite replication was assessed by two parameters: microscopic examination over time and parasite-specific real-time quantitative PCR (qPCR). Morphological differences and parasite population densities were visualized in all groups at 2, 24, 48 and 72 hpi by light microscopy. For qPCR, samples from each group were collected at 24, 48 and 72 hpi. DNA extraction was carried out using the QIAamp DNA Mini Kit® (Qiagen, Hilden, Germany) according to the manufacturer's protocol for cell cultures. The *T. gondii*-specific 529-bp repeat element was used to detect replication in a probe-based qPCR [35]. Standard curve samples were generated by gradient 10-fold dilutions of 10^7 tachyzoites to obtain absolute DNA copy numbers for *T. gondii* amplification. Replication of *E. tenella* was measured by ITS1 fragment quantification in a SYBR Green-based PCR as described before [36]. pSCA-17 plasmid standard dilutions were prepared as measure for the relative copy number of *E. tenella* DNA as described by Thabet et. al. [37]. The qPCRs were carried out in a Stratagene MX3000P cycler (Stratagene, La Jolla, USA). The cycling program for *T. gondii* detection included 95 °C for 15 min (initial denaturation), followed by 40 cycles of 95 °C for 15 s (denaturation), 60 °C for 1 min (annealing), and 72 °C for 15 s (extension). The cycling program for *E. tenella* was performed as follows: 95 °C for 5 min (initial denaturation), followed by 40 cycles of 95 °C for 30 s (denaturation), 62 °C for 20 s (annealing), and 72 °C for 20 s (extension). For *E. tenella*, a subsequent melting curve analysis (95 °C for 1 min, 62 °C for 30 s and 95 °C for 30 s) was applied to create the dissociation curve and ensure amplicon consistency. Data represent the mean of three replicates with an acceptable standard deviation for Ct values of less than 0.5.

Immune fluorescence assay (IFA) and confocal laser scanning microscopy (CLSM)

In addition to light microscopy, CLSM (TCS-SP8, Leica, Bensheim, Germany) was applied to observe the morphology of the infected cell cultures, for localization of parasites within the host cells, and to estimate the extent of intracellular replication. Therefore, 2×10^6 PBMC per well were cultivated in 8-well chamber slides (Ibidi, Martinsried, Germany) for 4 days to grow pure cultures of primary macrophages. Infection conditions were the same as mentioned above for all infection groups (TH, TL, EH, EL, CI and NC). For CLSM, cultures were fixed with methanol for 10 min before further processing. 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA) was used to stain cell nuclei. Cell imaging was carried out by Leica Application Suite X (LAS X, Leica Microsystems, Wetzlar, Germany).

Cytokine analysis

Samples were collected at 24, 48 and 72 hpi, and stored at -80 °C. Six cell culture replicates per infection group were analyzed for each time point. RNA was extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA was measured using a NanoPhotometer NP80 (Implen, Munich, Germany). The complementary DNA (cDNA) was synthesized using the Revert-Aid® first strand cDNA synthesis kit (Thermo Fisher Scientific, Darmstadt, Germany), according to the manufacturer's instructions. Briefly, 10 ng/µl total RNA were combined with 4 µl 5× reaction buffer, 1 µl RiboLock RNase Inhibitor (20U/µl), 2 µl 10mM dNTP Mix, 1 µl RevertAid M-MuLV RT (200U/µl), 1 µl of Oligo dT Primer (100 µM) and RNase-free water to a total volume of 20 µl. The mixture was incubated at 42 °C for 60 min and the reaction was terminated by heating at 70 °C for 5 min.

The mRNA expression levels of chicken host cell cytokines were measured by reverse transcription quantitative PCR (RT-qPCR). Specific sequences of the primers for TNF-α, IL-6, IL-10, IL-12, IFN-γ and iNOS cDNA amplification were selected (Table 1). Data normalization was performed based on the two chicken cell housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydrogenase (G6PDH) as described before [38]. For qPCR assay, 10 µl SYBR Green master mix (Thermo Fisher Scientific, Germany), 6.6 µl water, 0.4 µl ROX solution, 2 µl cDNA template, 0.5 µl forward primer and 0.5 µl reverse primer were used per reaction. The cycling program included 95 °C for 10 min (initial denaturation), followed by 40 cycles of 95 °C for 30 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 1 min (extension). A subsequent melting curve program (95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s) was applied to create the dissociation curve for each PCR run.

Statistics

Relative parasite DNA copy numbers as derived from qPCR results were calculated and presented as mean value (n = 3) for each time point after infection. Cytokines were quantified from RT-qPCR results using qBase

Table 1 Sequence of the primers used for chicken cytokine analysis by RT-qPCR

RNA target	Accession No.	Primer sequences (5'-3')		Reference
		Forward	Reverse	
GAPDH	K01458	GGTGGTGCTAAGCGTGTTAT	ACCTCTGTCATCTCTCTCCACA	[38]
G6PDH	AI981686	CGGGAACCAAATGCACTTCGT	CGCTGCCGTAGAGGTATGGGA	[64]
IFN-γ	Y07922	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC	[38]
IL-6	AJ309540	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTTCT	[38]
IL-10	AJ621614	CGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC	[38]
IL-12	NM_213571	AGACTCCAATGGGCAAATGA	CTCTTCGGCAAATGGACAGT	[38]
TNF-α	AY765397.1	CTTCTGAGGCATTTGGAAGC	ACTGGGCGGTCATAGAACAG	[65]
iNOS	U46504	TGGGTGGAAGCCGAAATA	GTACCAGCCGTTGAAAGGAC	[38]

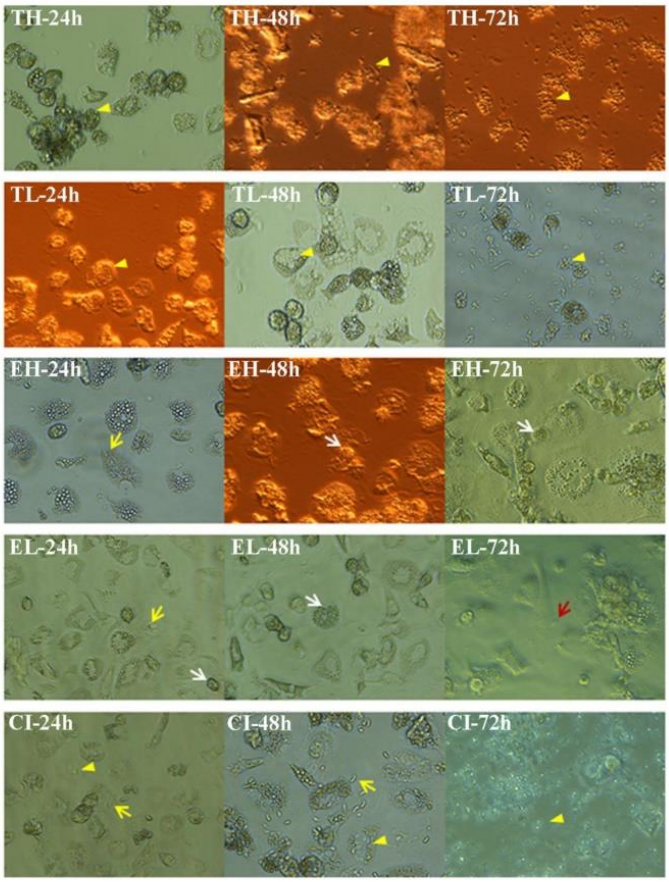

Fig. 1 Morphological visualization of primary macrophage cell cultures at 24, 48, 72 hpi by light microscopy. Yellow arrowhead: *T. gondii* tachyzoites; yellow arrow, *E. tenella* sporozoites; white arrow, *E. tenella* meronts; red arrow: *E. tenella* merozoites. *Abbreviations*: TH, high-dose infection with *T. gondii*; TL, low-dose infection with *T. gondii*; EH, high-dose infection with *E. tenella*; EL, low-dose infection with *E. tenella*; CI, co-infected group. Negative control group (NC) not shown

Plus 2.3 (Biogazelle NV, Belgium). Reference target stability and cytokine X-fold change differences between the infection groups were analyzed. X-fold change of cytokine expression was also calculated in comparison to the NC group data for each respective time point. Non-parametric Kolmogorov-Smirnov test was performed to test for normal distribution of data. Thereafter, the cytokine data were analysed statistically by ANOVA followed by Student's t-test (SPSS version 20 ®, IBM, New York, USA). Data are presented as mean ± standard error of the mean (SEM). A P-value < 0.05 is considered as statistically significant.

Results

Visual observation by light microscopy and CLSM

All infection groups were examined by light microscopy at four time points after infection (Fig. 1 and Table 2). At 2 hpi, invasion and adherence of *E. tenella* sporozoites were more obvious than for *T. gondii* tachyzoites in mono- and co-infected cell cultures (data not shown). Macrophage vacuolization occurred 24 hpi in the CI and *Toxoplasma* groups (TL and TH), but to a lesser extent in the *Eimeria* groups (EL and EH). Meanwhile, most of the *E. tenella* sporozoites remained in the intracellular sporozoite stage with low numbers of meronts appearing in both *Eimeria*-infected groups (EL and EH) and the CI group. At 48 hpi, many macrophages were detached in the infected cultures of TH group and CI group. There was a large number of free tachyzoites in the TH group with microscopic findings similar to 72 hpi in TL group and CI group. However, less free tachyzoites were visualized in TL and CI groups at 48 hpi. After 72 h, low numbers of *E. tenella* second generation of merozoites were found in the mono-infection groups EL and EH occasionally. By light microscopy, lowest numbers of macrophages were counted at 48 hpi. Almost no intact-appearing macrophage could be observed in *T. gondii* infection groups and co-infected group CI at 72 hpi.

In CLSM experiments using fluorescing transgenic parasites, it was observed that in co-infected macrophage cultures *T. gondii* partially replicated within host cells that also harbored *E. tenella* (Fig. 2a) from 24 hpi onwards. At 48 hpi, *T. gondii* meront numbers dominated over *E. tenella* meront numbers in the co-infected cell cultures of group CI (Fig. 2b). Host cell aggregation was seen in parallel with replication of *T. gondii* from 24 hpi.

Assessment of parasite replication by qPCR

DNA copy quantities representing *T. gondii* tachyzoites and *E. tenella* (all stages) in all samples were measured by qPCR. The average parasite replication dynamics were different in mono-infected groups compared to the CI group for both parasites (Fig. 3). In mono-infected groups, qPCR results indicated that *T. gondii* was able to replicate considerably in infected chicken macrophages of groups TH and TL over the study period. In the TH group, a final decrease in *T. gondii* stage numbers was seen 72 h.

Positive control MDBK cultures

Both parasites could be demonstrated in infected MDBK monolayers. Generally, in co-infected MDBK cell populations, *T. gondii* parasite numbers were slightly higher in comparison to mono-infected cultures as assessed by qPCR. However, no significant difference between

Table 2 Light microscopy findings by infection group and time point compared to uninfected NC group

	2 hpi			24 hpi			48 hpi			72 hpi		
	EH	**EL**	**CI**	**EH**	**EL**	**CI**	**EH**	**EL**	**CI**	**EH**	**EL**	**CI**
Attached macrophages	+++	+++	+++	+++	+++	++	+++	+++	++	++	++	+
Macrophage vacuolization	-	-	+	++	+	++	+	+	+++	++	++	+++
Free Et[a] sporozoites	+	+	+	-	-	-	-	-	-	-	-	-
Intracellular Et [a] sporozoites	+	+	+	+	+	+	-	-	-	-	-	-
Intracellular meronts	-	-	-	+	+	++	++	++	++	++	++	++
Et [a] merozoites	-	-	-	-	-	-	-	-	-	+	+	-[c]
	TH	**TL**	**CI**	**TH**	**TL**	**CI**	**TH**	**TL**	**CI**	**TH**	**TL**	**CI**
Attached macrophages	+++	+++	+++	++	+++	++	+	++	++	-	+	+
Macrophage vacuolization	++	+	+	++	+	++	+++	++	+++	-	+++	+++
Free Tg[b] tachyzoites	+++	++	++	+	-	-	+++	+	+	+	+++	+++
Intracellular Tg[b] tachyzoites	+	+	+	++	+	+	+	++	++	+	++	+++

Findings were scored semiquantitatively (-, not observed; +, low amounts; ++, moderate amounts; +++, high amounts). Infection groups: TH, high-dose infection with *T. gondii*; TL, low-dose infection with *T. gondii*; EH, high-dose infection with *E. tenella;* EL, low-dose infection with *E. tenella*, CI co-infected group.
[a]Et, *E. tenella*
[b]Tg, *T. gondii*
[c]Not clearly observable due to the numerous replication of *T. gondii* tachyzoites presented in cell culture by light microscopy

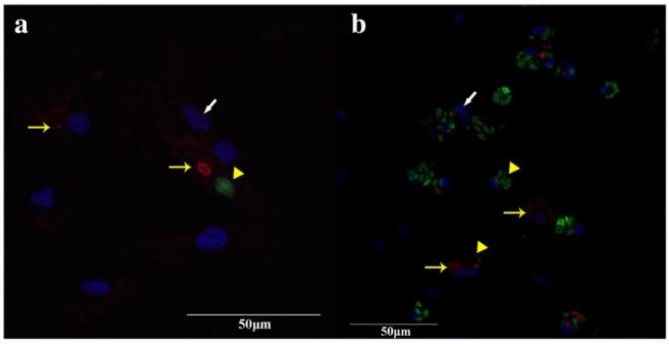

Fig. 2 Morphological visualization during co-infection of primary macrophage cell cultures by confocal laser scanning microscopy (CLSM). In co-infected macrophages, populations of *T. gondii* partially replicated in host cells which also stained positive for *E. tenella*. Nuclei of macrophages are stained blue (DAPI), *T. gondii* RH-GFP appear green and *E. tenella* Houghton-YFP red. **a** Cell culture co-infected with *T. gondii* and *E. tenella* at 24 hpi. **b** Cell culture co-infected with *T. gondii* and *E. tenella* at 48 hpi. White arrow: macrophage nuclei; yellow arrowhead: *T. gondii* tachyzoites; yellow arrow: *E. tenella* sporozoites or meronts

mono- and co-infection was observed for the replication of both parasites (data not shown).

Compared to macrophages, the stage numbers of both parasites at 72 hpi were significantly different in MDBK cells (Fig. 4). *Toxoplasma gondii* tachyzoites were not able to replicate sustainably in the MDBK co-infected culture and were not demonstrable after 24 hpi. The number of *T. gondii* tachyzoites was significantly lower in all mono- and co-infected MDBK cultures compared to the population in similarly infected macrophage cell cultures throughout the infection period. Conversely, replication of *E. tenella* was approximately quadrupled in relation to the infection dose at 24 hpi in co-infected MDBK cultures. By light microscopic observation, infected MDBK host cells showed less morphological alterations than macrophages. *Eimeria tenella* merozoites were clearly observed at 72 hpi.

Cytokine analysis

The relative mRNA expression of six cytokines was measured until 72 hpi by qPCR (Fig. 5) and compared with the uninfected group NC as X-fold changes. From the investigated cytokine panel, only IL-6 did not show relevant alterations in any group at any time point.

The measurement at 24 hpi showed a distinct elevation for iNOS and IFN-γ mRNA expression in EH and EL as well as CI groups ($F_{(5,\ 31)} = 4.81$, $P < 0.05$ compared to NC group). In contrast, TNF-α was only slightly increased. For groups TH and TL, iNOS and IFN-γ were not significantly altered though TNF-α and IL-10 were slightly increased. Only in group EL, an observable increase in IL-12 expression was induced ($F_{(4,\ 26)} = 3.76$, $P < 0.05$ compared to all other groups). No statistically significant differences were revealed between mono-infected groups

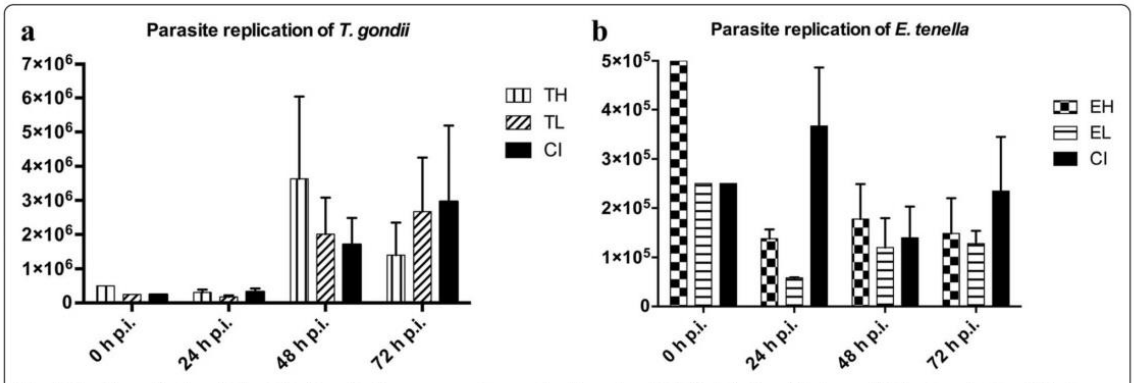

Fig. 3 Parasite replication during infection of primary macrophage cell cultures by qPCR. Co-infection (CI) showed highest replication 72 hpi. Parasite replication is shown as mean value with standard deviation ($n = 3$). **a** *T. gondii* replication in absence (TH, TL) or presence (CI) of *E. tenella*. *Abbreviations*: TL: low-dose *T. gondii* infection; TH high-dose *T. gondii* infection. **b** *E. tenella* replication in absence (EH, EL) or presence (CI) of *T. gondii*. *Abbreviations*: EH, high-dose *E. tenella* infection; EL: low-dose *E. tenella* infection; CI: co-infection

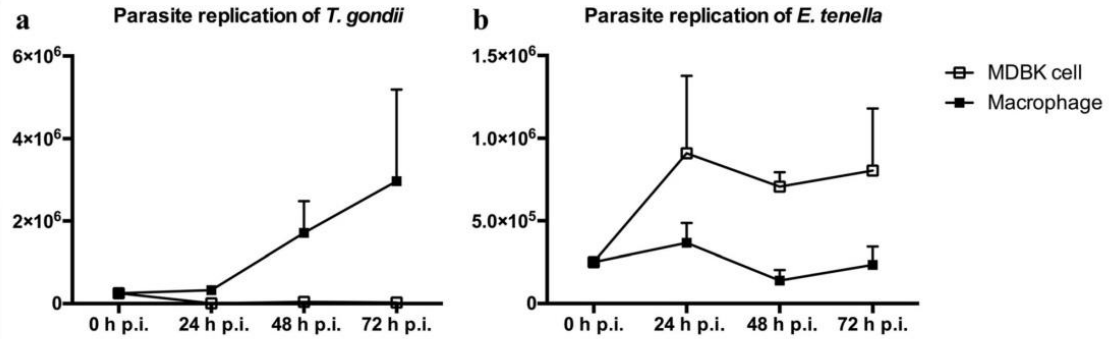

Fig. 4 Comparison of parasite replication during co-infection (CI group) of primary macrophages cell cultures and MDBK cells. Parasite replication was significantly different for both *T. gondii* and *E. tenella* in chicken macrophages compared to MDBK. **a** Parasite replication of *T. gondii*. **b** Parasite replication of *E. tenella* (mean and standard deviation, $n = 3$)

and the CI group for IFN-γ, IL-10, IL-12 or TNF-α, respectively.

At 48 hpi, IFN-γ mRNA expression was highest in group CI though group differences were not statistically significant. Significantly increased levels of IL-10 were observed in groups TH ($F_{(4,\ 26)} = 4.20$, $P = 0.020$ compared to TL) and CI ($F_{(4,\ 26)} = 4.20$, $P = 0.025$ compared to EL; $F_{(4,\ 26)} = 4.20$, $P = 0.105$ compared to TH). IL-12 was highly expressed in groups EH, EL, and TH. In contrast, IL-12 mRNA expression was not elevated in group CI ($F_{(4,\ 23)} = 2.31$, $P = 0.026$; $F_{(4,\ 23)} = 2.31$, $P = 0.029$ compared to EL and TH). Expression of iNOS mRNA peaked in group CI at 48 hpi ($F_{(4,\ 26)} = 2.32$, $P = 0.006$ compared to EL). TNF-α was not altered significantly by any infection mode until 48 hpi ($F_{(4,\ 24)} = 0.656$, $P > 0.05$ for all group comparisons).

At 72 hpi, IFN-γ mRNA expression levels were significantly increased for group EL ($F_{(4,\ 24)} = 1.64$, $P = 0.021$ compared to EH). IL-10 expression was higher in groups TH and CI ($F_{(4,\ 24)} = 1.20$, $P < 0.05$ compared to EH and EL). IL-12 expression was upregulated in all infected groups except for TL to a 2-fold to 3-fold extent. A moderate upregulation of iNOS as well as TNF-α expression were seen in all *T. gondii*-infected groups TH, TL and CI.

Discussion

Investigations into physiological alterations associated with experimental infections with mixed *Eimeria* spp. in chickens were previously published [39, 40]. However, to the best of our knowledge, there is only one published study of *Eimeria* spp. co-infection with *T. gondii* [19], probably because of the widely assumed sub-clinical character of toxoplasmosis in chickens [4]. The lack in primary clinical presentation does not necessarily imply that *T. gondii* infections may not influence the course of other infectious diseases in chickens. Since both pathogens, *Eimeria* spp. and *T. gondii*, are widely distributed in chicken flocks [4, 6, 41], this study focused on their potential interactions on the avian innate immune cells using primary macrophages as a model. Although the single published *in vivo* co-infection study of *T. gondii* and *E. tenella* did not demonstrate major mutual interaction in terms of inflammation and pathological findings, a significantly lower *E. tenella* oocyst excretion was observed in the co-infection group compared to *Eimeria* mono-infection group [19]. In the present *in vitro T. gondii* and *E. tenella* co-infection study, we found that there were various mutual effects between those parasites in chicken primary macrophages.

It was previously shown that chicken macrophages isolated from peritoneal exudates are capable of *E. tenella* sporozoite phagocytosis at 2–3 hpi [42]. There are convincing evidences that the clinical course and lethality of *E. tenella* infection were reduced by activated macrophages [43, 44]. On the other hand, sporozoites of *Eimeria* utilize chicken macrophages as transport cells [18], and tachyzoites of *T. gondii* can multiply in chicken primary macrophages [33, 45]. In the present study, we established a PBMC-derived macrophage culture for successful replication of both *E. tenella* and *T. gondii*. In general, *T. gondii* seemed to be better adapted to replication within chicken macrophages; however, *E. tenella* stages multiplied as well. As expected, both low-dose mono-infected groups EL and TL showed a slower and more sustainable increase in parasite numbers over time than the high-dose infected groups EH and TH. In a primary cell culture with a limited availability of host cells, it seems plausible that a high infection dose destroys host cells faster; thus rapidly hampering parasites replication than a low initial number of parasites. Although *E. tenella* showed an initial decrease in total gene copy numbers, this was followed by a distinct increase in co-infected cultures only. This may reflect that *T. gondii*

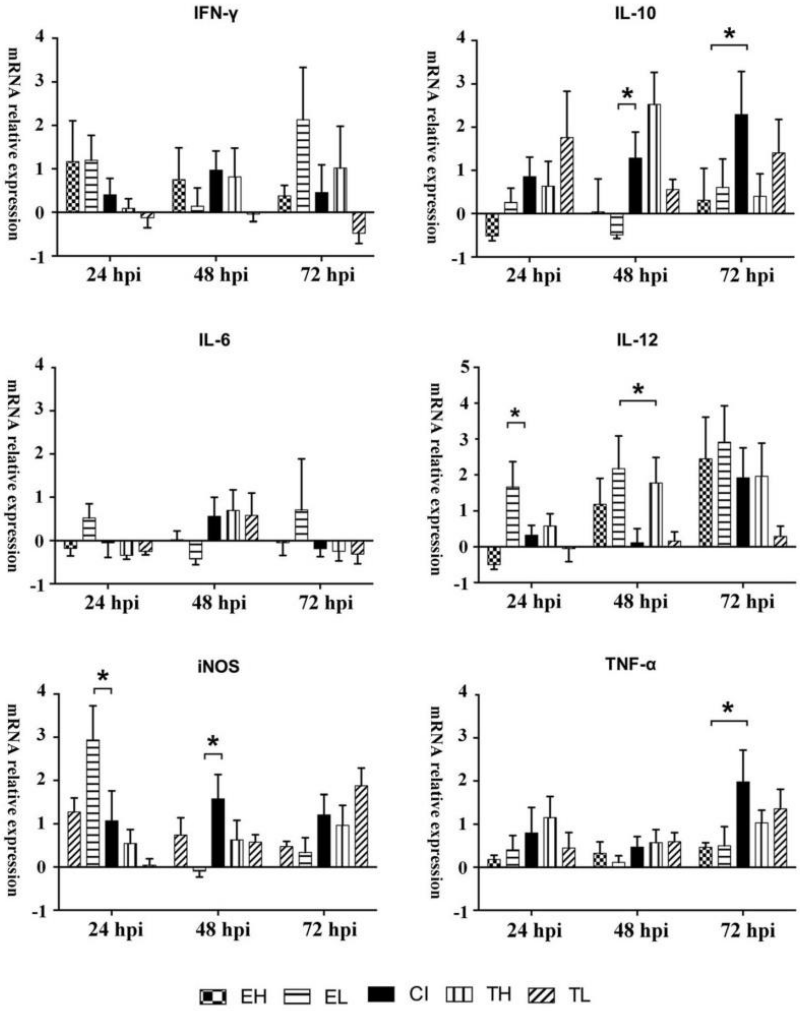

Fig 5 Macrophage mRNA relative cytokine expression by infection group. Normalization of data was performed on housekeeping genes GAPDH and G6PDH. The mRNA transcriptional levels of TNF-α, IL-6, IL-10, IL-12, IFN-γ, iNOS were determined by RT-qPCR. Displayed x-fold change values were calculated based on negative control (NC) group replicates ($n = 6$). Statistical comparison was performed between the CI group and other groups (*$P < 0.05$). *Abbreviations*: TH, high-dose infection with *T. gondii*; TL, low-dose infection with *T. gondii*; EH, high-dose infection with *E. tenella*; EL, low-dose infection with *E. tenella*; CI, co-infected group

supports *E. tenella* replication in macrophages; however, this hypothesis needs further investigation.

Mixed *Eimeria* spp. infections *in vivo* did not exert mutual effects on replication of three poultry *Eimeria* species [46]. In contrast, in our *in vitro* model we found that *E. tenella* and *T. gondii* displayed interactions that were most pronounced towards the end of the study (72 hpi). At this time, both *T. gondii* and *E. tenella* were replicating more strongly in co-infected cultures than in the mono-infected controls. This effect was particularly pronounced for *E. tenella*. However, the capability of *E. tenella* to invade macrophages was not influenced by co-infection with *T. gondii*. Light microscopic observations showed that first generation meronts were the dominating stages of *E. tenella* in all mono- and co-infected cultures. Unfortunately, the chosen co-infection model is not able to investigate a potential influence of *T. gondii* on sexual *E. tenella* development. It can be speculated that the increase in asexual *E. tenella* seen during co-infection might only be temporary and stage-related since another recent *in*

vivo investigation in our laboratory showed that a significantly low number of *E. tenella* oocysts were excreted by chicken co-infected with *T. gondii* than following *Eimeria* mono-infections [19]. In accord with our current findings, Hiob et al. [19] stated that the number of meronts in the intestine was not significantly altered by co-infection so the inhibiting effect of *T. gondii* on chicken *Eimeria* might be rather linked to the sexual development, which is not described to take place in macrophages.

The MDBK cell line was used to investigate growth and functions of *T. gondii* and *E. tenella in vitro* in various ways [47, 48]. Regardless of mono- or co-infection, we found that reproduction of *T. gondii* tachyzoites was broadly lower in MDBK cells than in chicken primary macrophages. In contrast to *E. tenella*, *T. gondii* replicated much more strongly in the macrophage culture. It appears possible that the cell metabolism of MDBK cells is altered at 41 °C, which is distinctly above the physiological bovine body temperature. However, no significant effect on parasite multiplication was found in co-infected MDBK cell cultures compared to primary chicken macrophages. This indicates an important role of the host cell type in induction or modulation of pathogen-pathogen interactions whereas the incubation temperature of 41 °C does not appear to play a major role in our model.

Chicken macrophages serve as phagocytes and regulatory immune cells. They produce cytokines, induce cytokine production in other immune cells, and destroy protozoans directly as part of the innate immune response [49, 50]. Although chickens are considered to be important natural hosts for *T. gondii* [4] and the only host species for *E. tenella* [51], immunoregulatory mechanisms by avian primary macrophages during simultaneous *T. gondii* and *E. tenella* infections are not sufficiently elucidated. We could demonstrate that, besides affecting the parasite replication potential, primary macrophage cell cultures reacted in different ways to both parasites during mono- and co-infections.

Macrophages are not the main source of IFN-γ but they are capable of IFN-γ expression [52]. IFN-γ plays an important role in the replication inhibition of the two investigated parasites [53, 54]. Five other cytokines produced by macrophages were included in our investigation: Th2-supporting IL-6, Th1-supporting IL-12, IFN-γ-inhibiting IL-10, and innate immune response-related iNOS and TNF-α. This panel was chosen because earlier studies clearly indicated the importance of those cytokines in coccidial infections [31, 46]. Single infections with *E. tenella* evoked host immune response which led to significant expression of cytokines such as IL-10 and IFN-γ in the ceca [46]. *In vivo*, it was demonstrated that mRNA expression of IFN-γ, IL-12 and IL-10 was distinctly upregulated in the ceca of chickens at the early stage of co-infection with *T. gondii* and *Eimeria* spp. [19]. In addition, high expression of those cytokines was observed in chickens co-infected with *E. tenella* and *Clostridium perfringens* [55]. Based on our observations in the co-infection model, using 12 hour intervals for cytokine expression measurements might be useful in the future to more comprehensively judge parasite-host cell interactions. However, we could observe several host cell reactions in this co-infected model.

In the present study, IFN-γ mRNA levels were upregulated mainly due to *E. tenella* infection, whereas co-infection seemed to suppress this Th1-related cytokine. Similar findings were recorded for IL-12 mRNA expression. Thus, it can be assumed that the adaptive Th1 response is rather suppressed than triggered in co-infected macrophages compared to mono-infections. In a recent study [56], a significant upregulation at 2 hpi and slight downregulation of IL-12 at 24 hpi were observed in a chicken macrophage cell line infected with *E. tenella* merozoites, which was diminishing over time. In contrast, IL-12 was upregulated by the end of the observation period in group CI, which might be related to the development of *E. tenella* meronts. Interestingly, in contrast to the cited *in vivo* studies [19, 55], we did not observe a distinct increase in IL-12 mRNA expression in co-infected macrophages. Therefore, we assume that not macrophages but other immune cell populations are responsible for the previously observed *in vivo* increase of IL-12 expression. In chickens, IL-6 mRNA expression was not significantly affected. This indicates that Th2 stimulation by macrophage-derived IL-6 does not play a major role in this infection mode.

IL-10 mRNA upregulation was observed mainly in T. *gondii* infection with a delayed peak in group CI that coincides with increased *E. tenella* replication. Since one of the multiple functions of IL-10 is the downregulation of IFN-γ expression, this corresponds clearly with the reduced transcription seen in CI. However, high expression of IL-10 in infected chickens is not associated to parasite multiplication or expression of cytokines like IFN-γ and IL-12 during *T. gondii* RH-infection [57]. Thus, it seems as if *in vivo* multiple immune cell populations influence the Th1 response to apicomplexan parasites, with macrophages being an important part of the complex innate immune response.

In spite of the relatively low IFN-γ levels in co-infected macrophages, reactive oxygen and nitrogen intermediates such as iNOS were produced in excess in these cells. This is interesting because IFN-γ is generally assumed to trigger the macrophages to produce these metabolites [31, 58]. It was previously [59, 60] demonstrated that *T. gondii* is altering NO production inhibition in chicken monocyte-derived macrophages and macrophage cell lines. In the present study, iNOS seemed to play a vital role in macrophage response

during co-infection (Fig. 5). Conversely, it was reported that iNOS was distinctly expressed during *E. tenella* infection *in vivo* or *ex vivo* [29]. This is in line with the general but moderate upregulation of iNOS mRNA expression in all infected groups seen in the present study.

Furthermore, we observed increased TNF-α expression in all infected groups, and this was particularly distinct in *T. gondii*-infected groups reaching a maximum at 72 hpi in the co-infected group. TNF-α increase was described before in *E. tenella*-infected chicken macrophages [32, 61]. Other authors [62, 63] stated a synergistic anti-*T. gondii* effect of TNF-α and IFN-γ. Our study demonstrated that co-infection significantly upregulated TNF-α while parasite replication increased more than in mono-infected cultures. We conclude that TNF-α production may either not be sufficient to counteract parasite replication in our model, especially in the presence of low IFN-γ levels, as indicated by Chang et al. [63], or that TNF-α mRNA expression does not exactly reflect actual TNF-α levels.

Conclusions

We demonstrated *in vitro* interactions between *T. gondii* and *E. tenella* in macrophages and MDBK cells. The findings of this study revealed that over a study period of 72 hpi, the replication of both parasites increased during co-infections compared to mono-infected cultures. Increased expression of IL-10 and TNF-α in co-infected cells demonstrates that interaction of both parasites is tightly linked to the host cell types and their various responses to infection. However, the present study leads to further questions. Additional experiments are needed to fully clarify the signaling pathways that are, e.g. leading to replication differences between mono- and co-infected cells. The presented findings are currently based solely on *in vitro* experiments that were chosen because of the defined conditions that allow for a more precise initial data interpretation. Future *in vivo* studies taking into account the natural interactions between different immune cell populations are needed to confirm our findings and their biological relevance, as well as to enable a betterunderstanding of the mechanisms of host-parasite and parasite-parasite interactions during co-infections. Additionally, investigations into subsequent infections with both pathogens will be helpful to estimate the relevance of non-simultaneous infections with both parasites that might be most relevant in the field.

Abbreviations

iNOS: inducible nitric oxide synthase; IL: Interleukin; IFN-γ: Interferon gamma; TNF-α: Tumor necrosis factor-α; TH: High-dose infection with *T. gondii*; TL: Low-dose infection with *T. gondii*; EH: High-dose infection with *E. tenella*; EL: Low-dose infection with *E. tenella*; CI: Co-infected group; hpi: hours post-infection; NC: Negative control group; MDBK: Madin-Darby Bovine Kidney; PBMC: Peripheral blood mononuclear cells; PBS: Phosphate-buffered saline; IFA: Immune fluorescence assay; CLSM: Confocal laser scanning microscopy

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

RZ designed the study, wrote the manuscript and performed the experiments. AT participated in the collection of parasites and chicken blood. LH and WZ analyzed data and helped to draft the manuscript. AD and BB critically revised the study design, data interpretation and manuscript. All authors read and approved the final manuscript.

Ethics approval

The animal experiments related to the blood sampling were approved by the responsible authorities (Landesdirektion Sachsen, Germany, trial registration number V13/10).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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References

1. Dubey JP, Beattie CP. Toxoplasmosis of Animals and Man. Boca Raton: CRC Press, Inc; 1988.
2. Unno A, Suzuki K, Xuan X, Nishikawa Y, Kitoh K, Takashima Y. Dissemination of extracellular and intracellular *Toxoplasma gondii* tachyzoites in the blood flow. Parasitol Int. 2008;57:515–8.
3. Dubey JP, Salant H, Sreekumar C, Dahl E, Vianna MCB, Shen SK, et al. High prevalence of *Toxoplasma gondii* in a commercial flock of chickens in Israel, and public health implications of free-range farming. Vet Parasitol. 2004;121:317–22.
4. Dubey JP. *Toxoplasma gondii* infections in chickens (*Gallus domesticus*): prevalence, clinical disease, diagnosis and public health significance. Zoonoses Public Health. 2010;57:60–73.
5. Anderson WI, Giambrone JJ, Fletcher OJ Jr, Eidson CS, Reid WM. Demonstration of *Eimeria tenella* in bursa of Fabricius of chickens. Avian Dis. 1976;4:752–5.
6. Dalloul RA, Lillehoj HS. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Rev Vaccines. 2006;5:143–63.
7. Hotop A, Buschtons S, Bangoura B, Zoller B, Koethe M, Spekker-Bosker K, et al. Humoral immune responses in chickens and turkeys after infection with *Toxoplasma gondii* by using recombinant antigens. Parasitol Res. 2014; 113:1473–80.
8. Chapman HD. Milestones in avian coccidiosis research: a review. Poult Sci. 2014;93:501–11.

9. Milanova A, Santos R, Lashev L, Koinarski V, Fink-Gremmels J. Influence of experimentally induced *Eimeria tenella* infection on gene expression of some host response factors in chickens. Bulg J Vet Med. 2016;19:47–56.
10. Soares Medeiros LC, Gomes F, Maciellr SSH, Docampo R, Moreno S, et al. Volutin granules of *Eimeria* parasites are acidic compartments and have physiological and structural characteristics similar to acidocalcisomes. J Eukaryot Microbiol. 2011;58:416–23.
11. Rose ME, Lee DL. Interactions *in vitro* between sporozoites of *Eimeria tenella* and host peritoneal exudate cells: electron microscopal observations. Z Parasitenk. 1977;54:1–7.
12. Tang X, Yin G, Qin M, Tao G, Suo J, Liu X, Suo X. Transgenic *Eimeria tenella* as a vaccine vehicle: expressing TgSAG1 elicits protective immunity against *Toxoplasma gondii* infections in chickens and mice. Sci Rep. 2016;6:29379.
13. Zou J, Huang X-X, Yin G-W, Ding Y, Liu X-Y, Wang H, et al. Evaluation of *Toxoplasma gondii* as a live vaccine vector in susceptible and resistant hosts. Parasit Vectors. 2011;4:168.
14. Mason S, Dubey JP, Smith JE, Boag B. *Toxoplasma gondii* coinfection with diseases and parasites in wild rabbits in Scotland. Parasitology. 2015;142:1415–21.
15. Black CM. Co-infection of macrophages modulates interferon gamma and tumor necrosis factor-induced activation against intracellular pathogens. J Exp Med. 1990;172:977–80.
16. Hériveau C, Dimier-Poisson I, Lowenthal J, Naciri M, Quéré P. Inhibition of *Eimeria tenella* replication after recombinant IFN-γ activation in chicken macrophages, fibroblasts and epithelial cells. Vet Parasitol. 2000;92:37–49.
17. Quéré P, Pierre J, Hoang M-D, Esnault E, Domenech J, Sibille P, Dimier-Poisson I. Presence of dendritic cells in chicken spleen cell preparations and their functional interaction with the parasite *Toxoplasma gondii*. Vet Immunol Immunopathol. 2013;153:57–69.
18. Van Doorninck WM, Becker ER. Transport of sporozoites of *Eimeria necatrix* in macrophages. J Parasitol. 1957;43:40–4.
19. Hiob L, Koethe M, Schares G, Goroll T, Daugschies A, Bangoura B. Experimental *Toxoplasma gondii* and *Eimeria tenella* co-infection in chickens. Parasitol Res. 2017;116:3189–203.
20. Malkwitz I, Berndt A, Zhang R, Daugschies A, Bangoura B. Replication of *Toxoplasma gondii* in chicken erythrocytes and thrombocytes compared to macrophages. Parasitol Res. 2017;116:123–31.
21. Zhou Z, Wang Z, Cao L, Hu S, Zhang Z, Qin B, et al. Upregulation of chicken TLR4, TLR15 and MyD88 in heterophils and monocyte-derived macrophages stimulated with *Eimeria tenella in vitro*. Exp Parasitol. 2013;133:427–33.
22. Denkers EY, Kim L, Butcher BA. In the belly of the beast: Subversion of macrophage proinflammatory signaling cascades during *Toxoplasma gondii* infection. Cell Microbiol. 2003;5:75–83.
23. Hong YH, Lillehoj HS, Lee SH, Dalloul RA, Lillehoj EP. Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. Vet Immunol Immunopathol. 2006;114:209–23.
24. Laurent F, Mancassola R, Lacroix S, Menezes R, Naciri M. Analysis of chicken mucosal immune response to *Eimeria tenella* and *Eimeria maxima* infection by quantitative reverse transcription-PCR. Infect and Immun. 2001;69:2527–34.
25. Ovington KS, Alleva LM, Kerr EA. Cytokines and immunological control of *Eimeria* spp. Int J Parasitol. 1995;25:1331–51.
26. Lyons RE, Anthony JP, Ferguson DJP, Byrne N, Alexander J, Roberts F, Roberts CW. Immunological studies of chronic ocular toxoplasmosis: up-regulation of major histocompatibility complex class I and transforming growth factor β and a protective role for interleukin-6. Infect Immun. 2001;69:2589–95.
27. Lynagh GR, Bailey M, Kaiser P. Interleukin-6 is produced during both murine and avian *Eimeria* infections. Vet Immunol Immunopathol. 2000;76:89–102.
28. Beaman MH, Hunter CA, Remington JS. Enhancement of intracellular replication of *Toxoplasma gondii* by IL-6. Interactions with IFN-gamma and TNF-alpha. J Immunol. 1994;153:4583–7.
29. Lillehoj HS, Li G. Nitric oxide production by macrophages stimulated with coccidia sporozoites, lipopolysaccharide, or interferon-γ, and its dynamic changes in SC and TK strains of chickens infected with *Eimeria tenella*. Avian Dis. 2004;48:244–53.
30. Allen PC. Nitric oxide production during *Eimeria tenella* infections in chickens. Poultry Sci. 1997;76:810–3.
31. Lieberman LA, Hunter CA. The role of cytokines and their signaling pathways in the regulation of immunity to *Toxoplasma gondii*. Int Rev Immunol. 2009;21:373–403.
32. Zhang S, Lillehoj HS, Ruff MD. *In vivo* role of tumor necrosis-like factor in *Eimeria tenella* infection. Avian Dis. 1995;39:859–66.
33. Malkwitz I, Berndt A, Daugschies A, Bangoura B. Long-term investigations on *Toxoplasma gondii*-infected primary chicken macrophages. Parasitol Res. 2013;112:3115–22.
34. Ekert J, Braun R, Shirley MW, Courdet P. Biotechnology Guidelines on Techniques in Coccidiosis Research. European Coorperation in the field of and Technical Research. COST. 1992;89:220–1.
35. Edvinsson B, Lappalainen M, Evengård B. Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis. Clin Microbiol Infect. 2006; 12:131–6.
36. Kawahara F, Taira K, Nagai S, Onaga H, Onuma M, Nunoya T. Detection of five avian *Eimeria* species by species-specific real-time polymerase chain reaction assay. Avian Dis. 2008;52:652–6.
37. Thabet A, Alnassan AA, Daugschies A, Bangoura B. Combination of cell culture and qPCR to assess the efficacy of different anticoccidials on *Eimeria tenella* sporozoites. Parasitol Res. 2015;114:2155–63.
38. Park SS, Lillehoj HS, Allen PC, Park DW, FitzCoy S, Bautista DA, Lillehoje EP. Immunopathology and cytokine responses in broiler chickens coinfected with *Eimeria maxima* and *Clostridium perfringens* with the use of an animal model of necrotic enteritis. Avian Dis. 2008;52:14–22.
39. Jenkins M, Allen P, Wilkins G, Klopp S, Miska K. *Eimeria praecox* infection ameliorates effects of *Eimeria maxima* infection in chickens. Vet Parasitol. 2008;155:10–4.
40. Jenkins M, Fetterer R, Miska K. Co-infection of chickens with *Eimeria praecox* and *Eimeria maxima* does not prevent development of immunity to *Eimeria maxima*. Vet Parasitol. 2009;161:320–3.
41. Shirley MW, Smith AL, Tomley FM. The biology of avian *Eimeria* with an emphasis on their control by vaccination. Adv Parasitol. 2005;60:285–330.
42. Long PL, Rose ME. Growth of *Eimeria tenella in vitro* in macrophages from chicken peritoneal exudates. Z Parasitenk. 1976;48:291–4.
43. Lee E-H, Al-Izzi SA. Selective killing of macrophages in the peritoneal cavity by carrageenan and its effect on normal infection of *Eimeria tenella* in chickens. Avian Dis. 1981;25:503–12.
44. Lillehoj HS, Kang SY, Keller L, Sevoian M. *Eimeria tenella* and *E. acervulina*: lymphokines secreted by an avian T cell lymphoma or by sporozoite-stimulated immune T lymphocytes protect chickens against avian coccidiosis. Exp Parasitol. 1989;69:54–64.
45. Meirelles MN, de Souza W. Killing of *Trypanosoma cruzi* and *Leishmania mexicana*, and survival of *Toxoplasma gondii*, in chicken macrophages *in vitro*. J Submicrosc Cytol. 1985;17:327–34.
46. Cornelissen JBWJ, Swinkels WJC, Boersma WA, Rebel JMJ. Host response to simultaneous infections with *Eimeria acervulina*, *maxima* and *tenella*: a cumulation of single responses. Vet Parasitol. 2009;162:58–66.
47. Appleford PJ, Smith JE. *Toxoplasma gondii*: the growth characteristics of three virulent strains. Acta Trop. 1997;65:97–104.
48. Thabet A, Zhang R, Alnassan A-A, Daugschies A, Bangoura B. Anticoccidial efficacy testing: *in vitro Eimeria tenella* assays as replacement for animal experiments. Vet Parasitol. 2017;233:86–96.
49. Qureshi MA, Heggen CL, Hussain I. Avian macrophage: effector functions in health and disease. Dev Comp Immunol. 2000;24:103–19.
50. Dalloul RA, Bliss TW, Hong Y-H, Ben-Chouikha I, Park DW, Keeler CL, Lillehoj HS. Unique responses of the avian macrophage to different species of *Eimeria*. Mol Immunol. 2007;44:558–66.
51. Blake DP, Clark EL, Macdonald SE, Thenmozhi V, Kundu K, Garg R, et al. Population, genetic, and antigenic diversity of the apicomplexan *Eimeria tenella* and their relevance to vaccine development. Proc Natl Acad Sci USA. 2015;112:E5343–50.
52. Gessani S, Belardelli F. IFN-γ expression in macrophages and its possible biological significance. Cytokine Growth Factor Rev. 1998;9:117–23.
53. Dimier-Poisson IH, Soundouss Z, Naciri M, Bout DT, Quéré P. Mechanisms of the *Eimeria tenella* growth inhibitory activity induced by concanavalin A and reticuloendotheliosis virus supernatants with interferon gamma activity in chicken macrophages and fibroblasts. Avian Dis. 1999;43:65–74.
54. Lillehoj HS, Choi KD. Recombinant chicken interferon-gamma-mediated inhibition of *Eimeria tenella* development *in vitro* and reduction of oocyst production and body weight loss following *Eimeria acervulina* challenge infection. Avian Dis. 1998;42:307–14.
55. Bangoura B, Alnassan AA, Lendner M, Shehata AA, Krüger M, Daugschies A. Efficacy of an anticoccidial live vaccine in prevention of necrotic enteritis in chickens. Exp Parasitol. 2014;145:125–34.
56. Chow Y-P, Wan K-L, Blake DP, Tomley F, Nathan S. Immunogenic *Eimeria tenella* glycosylphosphatidylinositol-anchored surface antigens (SAGs)

induce inflammatory responses in avian macrophages. PLoS One. 2011;6: e25233.
57. Wille U, Villegas EN, Striepen B, Roos DS, Hunter CA. Interleukin-10 does not contribute to the pathogenesis of a virulent strain of *Toxoplasma gondii*. Parasite Immunol. 2001;23:291–6.
58. Adams LB, Hibbs JB, Taintor RR, Krahenbuhl JL. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. J Immunol. 1990;144:2725–9.
59. DaMatta RA, Seabra SH, Manhães L, de Souza W. Nitric oxide is not involved in the killing of *Trypanosoma cruzi* by chicken macrophages. Parasitol Res. 2000;86:239–43.
60. Guillermo L, DaMatta R. Nitric oxide inhibition after *Toxoplasma gondii* infection of chicken macrophage cell lines. Poult Sci. 2004;83:776–82.
61. Zhang S, Lillehoj HS, Ruff MD. Chicken Tumor Necrosis-Like Factor. 1. *In vitro* production by macrophages stimulated with *Eimeria tenella* or bacterial lipopolysaccharide. Poult Sci. 1995;74:1304–10.
62. de Titto EH, Catterall JR, Remington JS. Activity of recombinant tumor necrosis factor on *Toxoplasma gondii* and *Trypanosoma cruzi*. J Immunol. 1986;137:1342–5.
63. Chang HR, Grau GE, Pechere JC. Role of TNF and IL-1 in infections with *Toxoplasma gondii*. Immunology. 1990;69:33–7.
64. de Boever S, Vangestel C, de Backer P, Croubels S, Sys SU. Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. Vet Immunol Immunopathol. 2008;122:312–7.
65. Nang NT, Lee JS, Song BM, Kang YM, Kim HS, Seo SH. Induction of inflammatory cytokines and toll-like receptors in chickens infected with avian H9N2 influenza virus. Vet Res. 2011;42:64.

2.2.3 Manuscript 3: Apicomplexan co-infections impair with phagocytotic activity in avian macrophages.

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I. Concept

Runhui Zhang was responsible for the idea and design of this work with the critical evolution and inspection of research goal and aims by Berit Bangoura and Arwid Daugschies.

II. Investigation

Runhui Zhang conducted the research and investigation process including performing the experiments and data collection with the partial participation of Wanpeng Zheng (cell imaging).

III. Analysis

Runhui Zhang performed data analysis with the partial participation of Wanpeng Zheng (CLSM imaging analysis and statistical analysis).

IV. Manuscript

Runhui Zhang wrote the initial draft and prepared the published work with critical revision and writing improvement by Wanpeng Zheng, Berit Bangoura and Arwid Daugschies.

Apicomplexan co-infections impair with phagocytic activity in avian macrophages

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Abstract

Background: *Toxoplasma (T.) gondii* and *Eimeria (E.) tenella* are two common parasites in poultry. Mixed infections are likely to occur frequently in chickens due to the high prevalence of both pathogens. In this study, we investigated the co-occurrence of the two pathogens in the same immune-competent host cell population towards potential parasite-parasite interactions as well as altered patterns of parasite-host interactions.

Results: Primary macrophages from chicken whole blood were co-infected with *T. gondii* RH strain tachyzoites and *E. tenella* Houghton strain sporozoites *in vitro* for 24 hours. Through monitoring the uptake of pH-sensitive pHrodo™ Zymosan BioParticles ('Zymosan') by macrophages, we were able to create a three dimensional model and to analyze quantitatively phagocytosis using confocal laser scanning microscopy. Assessments of parasite populations by qPCR were performed at 2, 6, 12 and 24 h post infection (hpi). At 6 hpi, phagocytosis was inhibited in the *E. tenella* infected cultures while no inhibition of phagocytosis was observed due to *T. gondii*. *T. gondii* was capable of replication in cells engulfing 'Zymosan' after activation. Macrophage phagocytosis activity revealed more complex interactions during co-infection. At 12 and 24 hpi, phagocytosis response to 'Zymosan' was distinctly weaker in co-infected cells than in all other groups except for cells monoinfected with high doses of *E. tenella* at 24 hpi. By qPCR, significantly reduced numbers of both intracellular parasites were recorded (10-fold) in all infected groups at 2 hpi. At 12 hpi, the *T. gondii* population reached lowest values but dramatically increased by 24 hpi.

Conclusions: Replication of both parasites increased during co-infection. Our data confirm that macrophage phagocytosis is involved in the control of invasion by apicomplexan parasites in chicken which particularly applies to *E. tenella* infection and it was able to be altered by the co-existing parasites.

Keywords: Apicomplexa, co-infection, chicken macrophages

Background

Toxoplasma (T.) gondii and *Eimeria (E.) tenella* are two ubiquitous intracellular parasites in poultry and are both representatives of parasitic coccidia. *T. gondii* infection is often asymptomatic and reported in almost all the warm-blood animals (HARKER, et al. 2015). Chicken are considered as resistant host with high seroprevalence of *T. gondii* worldwide (DUBEY. 2010). In contrast, *E. tenella* is one of the most pathogenic protozoan parasites in chicken and may cause severe enteric diseases and lethality. Even sub-clinical enteric coccidiosis reduces economic productivity in chickens (DALLOUL and LILLEHOJ. 2006).

Chicken macrophages play an important role as part of the first barrier of the innate immune response against *T. gondii* and *E. tenella* (UNNO, et al. 2008; HÉRIVEAU, et al. 2000; BUMSTEAD and MILLARD. 1987; DALLOUL and LILLEHOJ. 2006). Macrophages mainly contribute to the clearance and destruction of both intracellular and extracellular pathogens through phagocytosis (DALLOUL, et al. 2007). However, *T. gondii* can survive by forming a specialized parasitophorous vacuole (PV) in phagocytes such as macrophages (SIBLEY. 1995). There is evidence that protozoan parasites residing within human or murine macrophages block phagosome-endosome fusion by *Leishmania* or phagosome acidification by *Toxoplasma* (DESJARDINS and DESCOTEAUX. 1997; GOREN. 1977). Additionally, *T. gondii* within the PV block lipopolysaccharide (LPS)-triggered IL-12 and TNF-α during macrophage phagocytosis (BUTCHER and DENKERS. 2002). Regarding *Eimeria,* a previous study into *E. bovis* infection in bovine calves indicates significant macrophage infiltration of the intestinal mucosa (TAUBERT, et al. 2009). In chickens, it was shown that the engulfment of sporozoites by macrophages occurred few hours after infection and sporozoites were more often located within or next to macrophages in previously naïve than in *E. tenella* immune chickens (CHALLEY and BURNS. 1959; VERVELDE, et al. 1996; COX. 2001). Chicken macrophages isolated from peritoneal exudates were shown to engulf *E. tenella* sporozoites at 2-3 hours post infection (hpi) (LONG and ROSE. 1976). Sporozoite-bearing macrophages have been reported to possibly transport *Eimeria* spp. sporozoites to other host cells (TROUT and LILLEHOI. 1993; CHALLEY and BURNS. 1959; VAN DOORNINCK and BECKER. 1957).

Several published studies investigated interaction of cells involved in innate immunity and *T. gondii* or *Eimeria* spp. during mono-infection, few have referred to mutual host-parasite or parasite-parasite interaction with these two coccidian in co-infection models (HIOB, et al. 2017). In fact, mixed infections appear to occur frequently in chickens as seroprevalence of *T. gondii* and *Eimeria* spp. is high, especially in free-ranging chickens (LEHMANN, et al. 2006; DEYAB and HASSANEIN. 2005; AL-GAWAD, et al. 2012). A recent case has reported in Scotland showed co-infection of *T. gondii* and *Eimeria stiedae* in a wild rabbit (MASON, et al. 2015). Field studies on co-infections in chickens are currently lacking, however, an experimental *in vivo* study points at putative interaction of *T. gondii* and *E. tenella* during co-infection (HIOB, et al. 2017).

In our recent *in vitro* study into co-infection of chicken macrophages with *T. gondii* and *E. tenella*, destruction of parasites mainly occurred before 24 hpi (ZHANG, et al. 2018). *E. tenella* displayed a tendency to increase replication during co-infection with *T. gondii* at 72 hpi in chicken primary macrophage cultures. Besides, at 2 hpi more intracellular sporozoites of *E. tenella* than *T. gondii* tachyzoites – if by phagocytosis or invasion – were detected (ZHANG, et al. 2018). The current study aims at enhancing our understanding in host-pathogen and pathogen-pathogen interaction in co-infected chicken primary macrophage cultures. By generating a 3D model, we studied the capacity of macrophage phagocytosis in co-infected and mono-infected cultures. In addition, the invasion and reproduction of both *E. tenella* and *T. gondii* which are likely related to phagocytosis were investigated in the early phase of *in vitro* infection.

Results

Phagocytosis in uninfected chicken primary macrophages

NC phagocytosis was evaluated at time points corresponding to hpi of the infected cultures that were kept in parallel (Fig 1). Thus hpi is also used to describe the effects observed in NC although these cells were not infected. During the 2 h of exposure to ‘Zymosan’, no detectable changes in morphology or signs of apoptosis were found by light microscopy (data not shown). At 2 hpi, more than 70 % of DAPI positive macrophages incorporated ‘Zymosan’ (Table 1). In contrast, LPS exposure at the beginning of the experiment resulted in a clearly lower proportion of activated cells

to 53.7 ± 2.8 % of DAPI positive macrophages. In group NC, 57.6 ± 2.0 % of cells were phagocytic until 6 hpi, and 56.9 ±2.5 % cells were revealed 'Zymosan' positive at 12 hpi. At 24 hpi, phagocytosis percentage decreased to the lowest observed value with 36.6 ± 1.2% (Table 1).

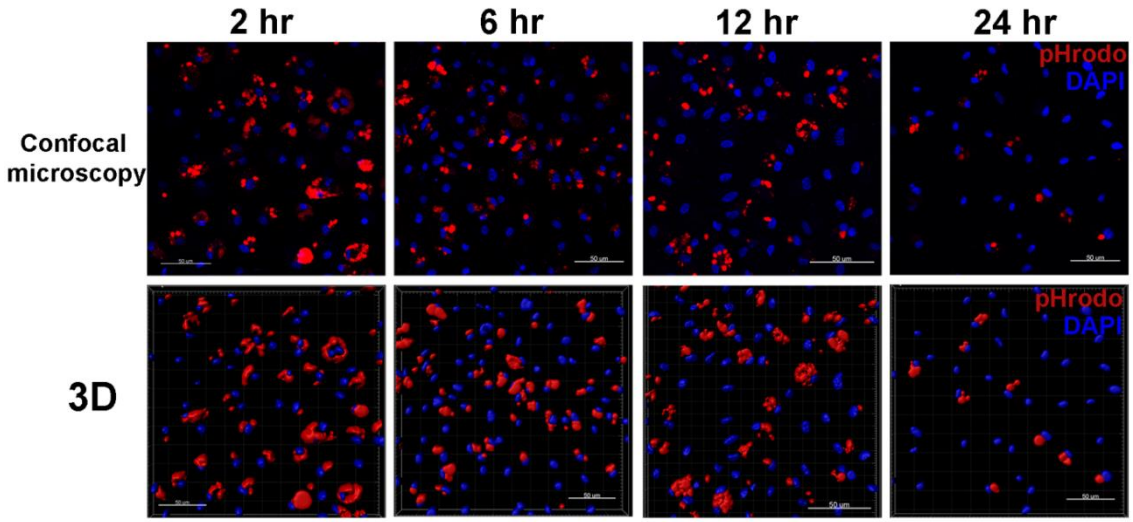

Fig 1 Phagocytosis activity of primary chicken macrophages at 2, 6, 12 and 24 hpi. Phagocytosis was determined after 2 h exposure to 'Zymosan' by cell imaging with. Staining: DAPI (cell nuclei, NucBlue™ Live ReadyProbes™ blue); pHrodo ('Zymosan' bioparticles, red). Confocal laser scanning microscopy (CLSM), 200x magnifications; 3D model generated by Imaris®

Table 2 Phagocyotosis of primary chicken macrophages was evaluated using pHrodo™ red Zymosan BioParticles

Further cultivation time point	[a]% of Phagocytic Macrophages	
	Mean	SD[b]
2 hr	71.96	4.83
6 hr	57.64	2.02
12 hr	56.86	2.49

24 hr	36.57	1.08
[c]LPS stimulation at 0 hr	53.65	2.83

[a]% of Phagocytic Macrophages= 100 x (total granulocytes 'Zymosan'/ total macrophage nuclei blue); Total macrophages with or without 'Zyomsan' were calculated by Imaris® Software.

[b]SD: standard deviation

[c]LPS: Lipopolysaccharide, 1 µg/ml, 1 hr activation, n = 3

Phagocytosis in infected macrophage cultures

Cell cultures were finally infected 4 days after onset of cultivation as listed in Table 2. At 2 hpi, all infected groups showed initial inhibition of phagocytosis, however, on variable levels (Fig 2a and Additional file 1). Inhibition was most pronounced in groups TH and EH, being significantly higher than in group CI ($p<0.005$). No additive effect was seen in phagocytosis inhibition by co-infection as compared to groups TL and EL that were mono-infected with the same dose of the respective parasite as used for co-infection of group CI.

Table 1 Infection groups

Infection	Group TH	Group TL	Group CI	Group EH	Group EL
Toxoplasma gondii tachyzoites	5×10^5	2.5×10^5	2.5×10^5	-	-
Eimeria tenella sporozoites	-	-	2.5×10^5	5×10^5	2.5×10^5

Over the following investigation period patterns of inhibition varied.

At 6 hpi, *E. tenella* infection inhibited macrophage phagocytosis in group EH and, less distinctly, in group EL, whereas *T. gondii* showed no inhibitory effect in groups TH and TL (Fig 2b). Likewise, no phagocytosis inhibition was observed in group CI. Compared to group CI, both groups EH and EL displayed significantly higher inhibition values ($p < 0.005$ and $p < 0.05$, respectively).

At 12 hpi, Zymosan-induced activation of phagocytosis was similar to group NC in all four mono-infected groups (TH, TL, EH, EL). In contrast, group CI featured a remarkably reduced response to stimulation of phagocytosis, and inhibition values were significantly higher than in all other infection groups ($p < 0.005$ in comparison to TL, EH and EL and $p < 0.05$ in comparison to TH) (Fig 2c).

At 24 hpi, group CI remained at a high inhibition level although values were significantly lower than in group EH ($p < 0.005$) (Fig 2d). In contrast, groups TH and EL did not display reduced phagocytosis, and consequently inhibition values were negative and significantly lower than in group CI ($p < 0.005$ compared to TH and $p < 0.05$ compared to EL). Group TL showed only slight inhibition, although a statistical effect was seen in comparison to group CI ($p < 0.05$). Strikingly, highest phagocytosis inhibition values were observed in group EH, and the respective values were significantly higher than in group CI ($p < 0.005$)

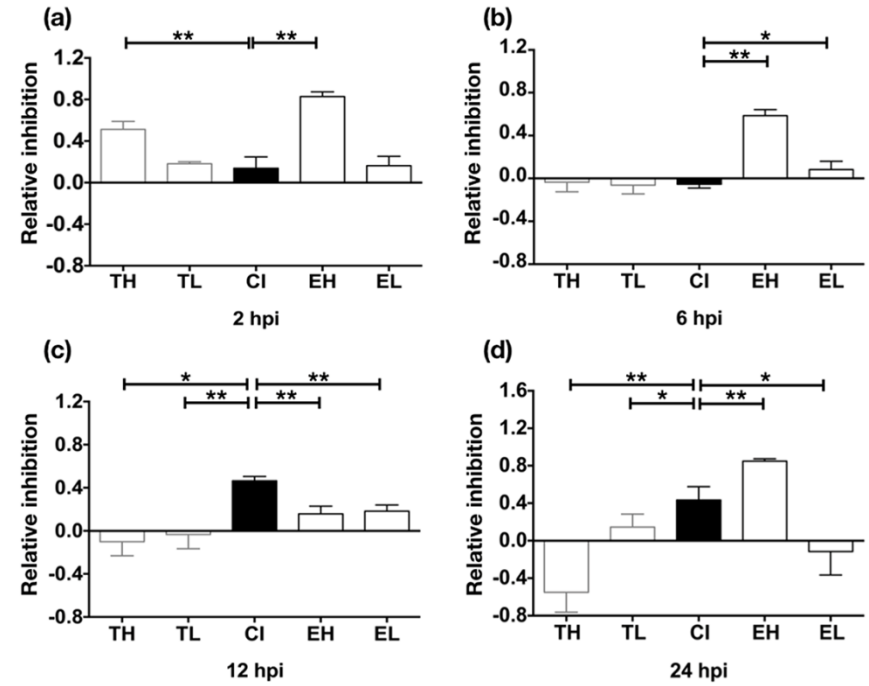

Fig 2 Relative inhibition of 'Zymosan' engulfment in chicken macrophages by mono- and co-infection until 24 hpi**.** Values represent mean values and standard deviation in relation to phagocytosis in group NC (uninfected, n = 6). (a) Relative inhibition at 2 hpi. (b) Relative inhibition at 6 hpi. (c) Relative inhibition at 12 hpi. (d) Relative inhibition at 24hpi. Asterisks (*$p < 0.05$, **$p < 0.005$) indicate a significant difference between groups (Mann Whitney U test; denomination of

groups: see Table 2)

CLSM

E. tenella meronts and replicating *T. gondii* tachyzoites were visible in all infected macrophage cultures at 12 hpi (Fig 3A). However, fewer intracellular *E. tenella* sporozoites or first generation of *E. tenella* meronts were observed than intracellular *T. gondii* in the respective cultures. 'Zymosan' positive cell was not visualized inside any co-infected cells of group CI.

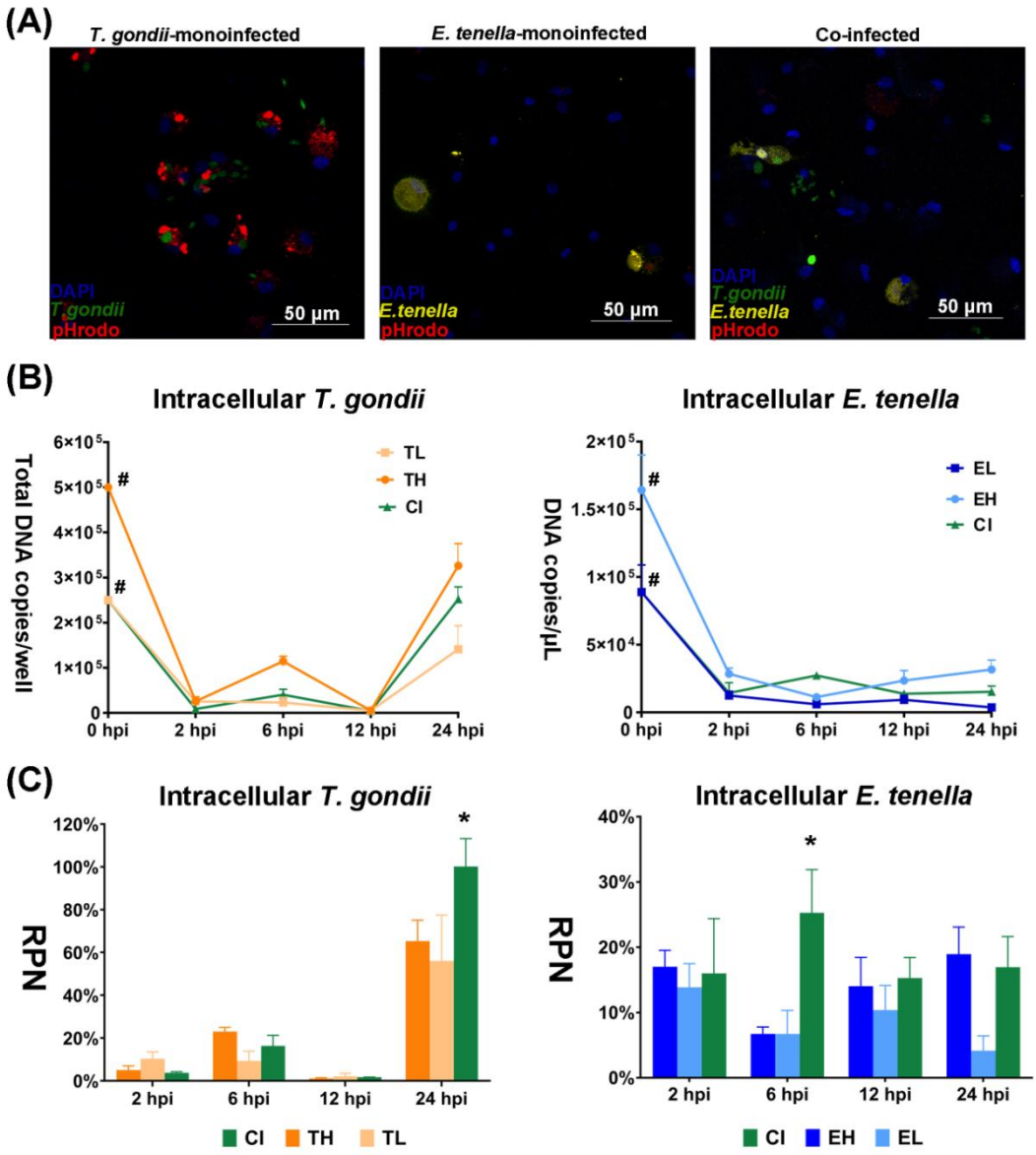

Fig. 3 Cell imaging at 12 hpi and assessment of parasite reproduction during infection until 24 hpi. (A) Cell imaging of parasite replication by CLSM at 12 hpi. DAPI: cell nuclei stained blue); pHrodo

(Zymosan bioparticles) stain red; T. gondii (tachyzoites, GFP, green); E. tenella (sporozoites and meronts, YFP, yellow). **(**B) Quantities of intracellular parasite estimated by qPCR. T. gondii tachyzoites are represented as total DNA copies/well and E. tenella (all stages) as DNA copies/µL. # represented DNA quantities of initial infection doses. (C) Relative parasite numbers (RPN) of T. gondii and E. tenella compared to the initial infection doses. RPN were calculated as mean value of parasite DNA copies with standard deviation (n=3 or 4). * $p < 0.05$ is considered a statistically significant difference between marked infected groups (based on Mann Whitney U test; denomination of groups**:** see Table 2).

Parasite replication

T. gondii

Following a rapid initial decrease, low *T. gondii* DNA copy numbers were observed in groups TH, TL, and CI until 12 hpi. However, the quantity of *T. gondii* DNA distinctly increased thereafter by 24 hpi. Interestingly, at the end of the experimental period group CI reached almost the level of the high dose mono-infected group (TH) although the infection dose applied was similar to group TL which obviously remained on a lower level of reproduction (Fig. 3B). Relative parasite numbers (RPN) amounted to about 5 % in group TH and 10 % in group TL at 2 hpi (Fig 3C). At this time, lowest RPN (3.6 ± 0.1 %) were observed in group CI, which were significantly lower as compared to group TL ($p < 0.05$). At 6 hpi, an increase in RPN was observed for group TH (22.9 ± 2.2%), followed by a transient decline at 12 hpi (1.1 ± 0.4%) and finally a steep increase at 24 hpi (65.3 ± 9.8%). In group TL, a similar trend was observed with comparatively low RPN values until 6 hpi (9.3 ± 4.4 %) and even a further reduction at 12 hpi (2.1 ± 1.4 %), followed by a very distinct increase at 24 hpi (56.4 ± 21.0 %).

In group CI, low RPN levels comparable to those recorded for the mono-infected groups TH and TL were observed with lowest values at 12 hpi (1.7 ± 0.1%). However, at 24 hpi, RPN values of group CI (100.1 ± 13.1 %) clearly exceeded those of both mono-infected groups ($p < 0.05$). Based on the same initial *T. gondii* infection dose replication was almost twice as high in the co-infected group as in the mono-infected group TL at the termination of the experiment.

E. tenella

The quantity of intracellular *E. tenella* stages (sporozoites or first generation meronts) was reduced as compared to the initial dose at 2 hpi and remained low until the end of the experiment irrespective of the applied mono-infection dose (EL, EH) or co-infection (CI; Fig 3B) without any significant group effects.

Consequently, RPN values were low at 2 hpi with only 13.9 ± 3.6 % in group EL and 17.0 ± 2.5 % in group EH (Fig 3C). In both mono-infected groups RPN levels further decreased until 6 hpi (group EL: 6.7 ± 3.6 %; group EH: 6.7 ± 1.1 %). Until 12 hpi, RPN moderately increased in these two groups indicating moderately increased reproduction (group EL: 10.4 ± 3.8%; group EH: 14.0 ± 4.4%). This trend continued until 24 hpi in group EH (18.9 ± 4.3 %) whereas RPN values had decreased in group EL by then (4.2 ± 2.3%).

Initially group CI displayed similar RPN (16.0 ± 8.4 %) as the mono-infected groups. However, a very distinct peak of RPN values (25.3 ± 6.6 %) was noted in group CI at 6 hpi ($p < 0.05$). At 12 hpi and 24 hpi RPN of CI cultures were rather similar to those of the EH group (15.3 ± 3.1% and 17.0 ± 4.8 % at 12 hpi and 24 hpi, respectively) and, at 24 hpi, RPN of group CI, infected with the same *E. tenella* dose as cultures of group EL, were 4-fold higher than in the EL group.

Discussion

Macrophages are professional phagocytes and highly efficient at internalizing particles (ADEREM and UNDERHILL. 1999). Flow cytometry and pH-sensitive fluorescent particles have been broadly used to visualize and quantify phagocytosis by macrophage cell lines (PÉREZ-FLORES, et al. 2016) or non-adherent phagocytic cells (NEAGA, et al. 2013; GORDON, et al. 2017). However, a recent study (KAPELLOS, et al. 2016) indicated that primary macrophages showed higher sensitivity to external stimulation and could suffer more from stress than other cells during fluorescent staining or running on a flow cytometer. We made similar observations working with chicken primary macrophages (data not shown). Most phagocytosis assays were carried out in mammalian macrophages, so far. However, direct comparison of data from mammalian macrophage cultures to our study is of limited value because the avian immune system features complex differences to the

mammalian immune system, i.e. avian macrophages interact differently with pathogens (ERF. 2004). Only two recent reports were found on the investigation of phagocytosis in a chicken macrophage cell line (HD11 cells) (GARRIDO, et al. 2018; LEE, et al. 2018), but no studies in primary chicken macrophages have been published previously to the best of our knowledge.

In our experiments, we firstly investigated the capacity of cultured chicken primary macrophages to engulf 'Zymosan' at four observation periods up to 24 hpi. Macrophage showed great adaptation without observed apoptosis after applying 'Zymosan' following the manufacturer's instructions. Initial engulfment was efficient with on average 72 % of macrophages displaying phagocytosis at 2 hpi. However, macrophage activity decreased to 36.6 % until the end of the study period. This finding indicates that primary macrophage phagocytosis is generally declining over time. Compared to a recent study of Garrido et al. (GARRIDO, et al. 2018), showing that about 25% of HD 11 cells retain phagocytic activity induced by pH-sensitive bioparticles after 16 h mock treatment, our observed phagocytosis percentages are in a good range over the whole chosen study period.

The cell density of murine bone marrow-derived macrophages (BMDMs) and the duration of activation played a crucial role in uptake of fluorescent particles (KAPELLOS, et al. 2016). Therefore, for longer-term phagocytosis investigations in primary avian macrophages, optimization of cultivation and assay conditions may be crucial to improve phagocytosis capacity over a longer period of time. In our study, LPS polarization led to a reduction of the phagocytic macrophage activity by about 20%. This is in line with the findings in murine BMDMs, where it is assumed that cytokine expression changes can modulate their ability to engulf fluorescent particles (KAPELLOS, et al. 2016). Altogether, the model described here appears to be a suitable model to perform investigations into *T. gondii* and *E. tenella* infections including co-infection in chicken macrophages.

Pathogen-pathogen interaction is more and more recognized as an important factor in pathogenesis and disease outcome. For parasites, *in vivo* studies into enhanced interactions of pathogens co-existing in a host and evidence of competition were shown (CLARK, et al. 2016; ONAGA, et al. 1983; HIOB, et al. 2017). Regarding coccidia, clinical disease caused by *E. maxima* is ameliorated by concurrent *E. praecox* infections in chickens (JENKINS, et al. 2008). *Eimeria* replication in terms of oocyst excretion numbers does not seem to be influenced by co-infections with other

Eimeria species in most *in vivo* studies (CORNELISSEN, et al. 2009). However, a study into experimental *E. tenella* infections showed significantly lower oocyst excretion in a group co-infected with *T. gondii* than in an *Eimeria* mono-infected group (HIOB, et al. 2017).

In contrast, our previous long term *in vitro* study revealed that both *T. gondii* and *E. tenella* were multiplying more effectively in co-infected cultures than in mono-infected cultures over a period of 72 hpi (ZHANG, et al. 2018). Looking into the related impact on macrophage function as a crucial aspect of the innate immune response, we have identified phagocytosis, which is the major activity of chicken macrophages during pathogen exposure, to be severely impacted by *T. gondii* and *E. tenella* co-infections.

With regard to the mode of invasion, it has been demonstrated that a proportion of *T. gondii* may invade host cells actively while avoiding an efficient phagocytic response through formation of a parasitophorous vacuole (MORISAKI, et al. 1995; BUTCHER and DENKERS. 2002). For *E. bovis*, active invasion is negligible in bovine primary macrophages (TAUBERT, et al. 2009). For both *T. gondii* and *E. tenella*, phagocytosis by the host cell was identified earlier as the major route of pathogen entry (GOREN. 1977; ROSE and LEE. 1977a).

In our phagocytosis assay, all *T. gondii* or *E. tenella* mono-infected groups showed inhibition of 'Zymosan' engulfment at 2 hpi. Inhibition of phagocytosis in the high-dose infected groups (TH, EH) was more obvious than in low-dose infection groups (TL, EL). This is in line with a dose-dependent parasite replication (RPN) as observed for both parasites. Interestingly, co-infected macrophage cultures exhibited a significantly lower level of phagocytosis inhibition than seen in all mono-infection groups. Intracellular parasite quantification demonstrated that more than double *T. gondii* copies were detected in group TL whereas slightly lower *E. tenella* copy numbers were found in group EL compared with group CI, which shows that parasite replication is affected differently for *T. gondii* and *E. tenella* during co-infection. While *E. tenella* displays a higher initial incorporation rate upon co-infection, *T. gondii* appears to feature an impaired invasion rate. According to our recent light microscopical study (ZHANG, et al. 2018) and live cell imaging results (unpublished yet), most *E. tenella* sporozoites were capable at 2 hpi to enter macrophages while most *T. gondii* tachyzoites 'prefer' to adhere to the surface of a macrophage or stay free in the cell culture medium. One

previous *in vitro* study likewise reported the capacity of chicken macrophages to engulf *E. tenella* sporozoites at 2-3 hpi (LONG and ROSE. 1976). Another study showed that a high proportion of live *T. gondii* stayed loosely adherent to host cells without invading and inducing phagocytosis (MORISAKI, et al. 1995). On the other hand, a previous study showed that most *T. gondii* tachyzoites remained adherent to murine macrophages followed by phagocytic inhibition treatment (RYNING and REMINGTON. 1978). We speculate that the initially reduced *T. gondii* RPN in group CI compared to mono-infection groups may be that *T. gondii* endocytosis by macrophages was blocked on the host-cell side by phagocytosis-inhibited macrophages previously entered by *E. tenella*. However, further evidences need to support it by investigating the signal pathway interactions.

Entry of both *T. gondii* and *E. tenella* increased whereas no inhibition was seen on phagocytic capacity of macrophages in co-infection at 6 hpi. Interestingly, co-infection showed distinctly lower phagocytic inhibition but higher RPN of *E. tenella* than *E. tenella* mono-infection group. Meanwhile, phagocytic inhibition by mono-infection of both parasites likely revealed biphasic at 6 and 12 hpi in our study. Specifically, inhibition appeared mostly in the *E. tenella* infection. Due to lack of comparable research, we assumed that significant engulfment of *E. tenella* was associated possibly to the low phagocytosis-inhibited macrophages modulating by *T. gondii* infection.

Combining to our data of quantification and visualization, it appears that a small number of both parasites retained their capability to evade from macrophage phagocytosis and developed successfully while most were eliminated at 12 hpi. Similar results were seen before in mono-infected chicken primary macrophage cultures with the lowest parasite DNA copies of *T. gondii* at the same point. (MALKWITZ, et al. 2017). *T. gondii* and *E. tenella* stages were occasionally identified within the same individual macrophages, which is in line with findings from our previous study (ZHANG, et al. 2018). *E. tenella* exhibited higher initial infection efficiency than *T. gondii* as inferred from RPN at 12 hpi in relation to the applied infection dose. However, CLSM suggests that most of *E. tenella*-YFP failed to show fluorescence and little intracellular sporozoites or meronts were visualized during *Eimeria*-infection. The co-infected cell cultures showed distinctly lower activation by ‘Zymosan’ than mono-infected cultures at 12 hpi. Interestingly, phagocytic activity as measured by ‘Zymosan’ incorporation into macrophages was initially inhibited stronger in both groups EH and

TH than in the co-infected group CI which was exposed to both parasites at half the infection dose. This effect reversed at 12 hpi indicating that co-infection leads to more complex effects on a host culture than a mono-infection. Similar to the LPS polarization which was described earlier, co-infection likely triggered immune pathways in the cultures, and stronger so than mono-infections did. A previous study revealed increased expression of nitric oxide synthase (iNOS), IL-1β and decreased IL-10 occurred in chicken HTC macrophage infected with *E. tenella* merozoites at 12 hpi compared to 6 hpi (CHOW, et al. 2011). However, our recent study showed that T. *gondii* and *E. tenella* co-infection increased mRNA expression for both Th1- and Th2-related cytokines markedly, including Th2-related IL-10 (ZHANG, et al. 2018). It is well known that IL-10 inhibits nitric oxide formation in activated macrophages (HUANG, et al. 2002). Thus we deduce that weakening the phagocytic activity of the macrophage cultures may be associated to the alteration of innate immune response in co-infection, which needs to be further discussed.

Relative phagocytosis inhibition values at the end of our study period (24 hpi) were strongly increased in groups EH and CI, while TH cultures displayed significantly higher phagocytosis activity than uninfected cultures. Seemingly, *E. tenella* appears to be mainly responsible for the reduced macrophage phagocytosis that was observed in group CI. Both studied apicomplexan parasites have similar ways of host cell invasion and intracellular replication. Nevertheless, they exhibit different effects on chicken macrophage function related to innate immunity. It may be hypothesized from our data that parasite stage and replication as well as parasite adaption to its host may be involved in the observed infection related group differences. Follow-up studies including *in vivo* studies or immune intervention will be needed to investigate the underlying mechanism of phagocytosis inhibition (*E. tenella* and co-infections) or enhancement (*T. gondii* infections towards the end of the study).

Conclusion

This *in vitro* infection model of primary avian macrophages can provide an efficient and accurate way to further investigate mechanisms of host-parasite and parasite-parasite interaction during co-infections with pathogens such as monoxenous and heteroxenous coccidia. The current findings contribute to our understanding of macrophage modulation by intracellular parasites and

functionality at the early stage of infection with *T. gondii* and/or *E. tenella.*

Methods

Chicken primary macrophage isolation and culture

Chicken peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood of adult chickens, based on the established protocols kindly provided by Dr. Braukmann, Friedrich-Loeffler-Institute Jena, Germany. The animal experiments related to the blood sampling were approved by the responsible authorities (Landesdirektion Sachsen, Germany, trial registration number V13/10). 2 mL blood was mixed gently with 2 mL phosphate-buffered saline (PBS) containing 1 mg/mL gentamicin (Life technologies, Darmstadt, Germany). 2 mL Biocoll® separating solution (density 1,077 g/ml; Biochrom AG, Berlin, Germany) was used to separate the PBMCs by centrifugation at 250 x g for 45 min. Afterwards, the isolated PBMC were washed with 5 mL PBS once, followed by centrifugation at 350 x g for 30 min. The resulting pellet was washed and centrifuged (350 x g, 20 min) once with 5 mL pre-warmed, 41 ℃, RPMI-1640 medium (Sigma, Taufkirchen, Germany). Subsequently, 5 x 10^6 PBMC/well were resuspended in RPMI with 5% chicken serum and 5% fetal bovine serum, penicillin (100 U/mL, PAA), streptomycin (0.1 mg/mL, PAA), and amphotericin B (0.0025 mg/mL, PAA), and incubated in 24-well plates for 96 h (41 ℃, 5 % CO_2). The macrophages were purified by rinsing off non-adherent cells twice in PBS at 2 h and 24 h after seeding, and directly before infection.

Parasites

Genetically modified *T. gondii* RH-GFP tachyzoites (type I strain, kindly provided by Professor Dominique Soldati-Favre, University of Geneva Medical School, Switzerland) were harvested from infected human foreskin fibroblast (HFF) cultures. *E. tenella* Houghton-YFP strain (kindly provided by Professor Xun Suo, China Agricultural University, China) sporozoites were gained following an established protocol (THABET, et al. 2017) with slight improvement. Briefly, sporocysts were collected by mechanical destruction of the oocyst wall with 0.5 mm glass beads (BioSpec Products, Bartlesville, OK, USA). Sporocysts were incubated in 0.25% trypsin (w/v) (Carl Roth, Karlsruhe,

Germany) and 4% sodium taurocholic acid (w/v) (Sigma-Aldrich, Taufkirchen, Germany) at 41 ℃ for 90 min for excystation. Then, sterile pluriStrainer® 5 µm (pluriSelect Life science, Leipzig, Germany) was used to purify excysted sporozoites with 1% glucose in PBS at pH 7.4 (follow buffer).

Infection

Cell cultures were divided into six groups (n = 6 cultures per group) and infections were performed as previously described (ZHANG, et al. 2018). Briefly, infection groups were performed with a multiplicity of infection (MOI) of certain parasites per cell (Table 1): Group NC consisted of uninfected negative control PBMC cultures that were seeded 4 days before the start of the experiment. Infection groups were conducted as follows: Groups TH (MOI of 4 *T. gondii* tachyozites), TL (MOI of 2 *T. gondii* tachyozites), EH (MOI of 4 *E. tenella* sporozoites), EL (MOI of 2 *E. tenella* sporozoites), and CI (MOI of 2 *T. gondii* tachyozites and 2 *E. tenella* sporozoites). Groups were observed over a period of 24 h.

Phagocytosis assay

24-well plates for cell imaging (Bottom thickness: 170 µm, Cellvis, CA, USA) were used to assess macrophage phagocytosis according to the manufacturer's protocol. Briefly, 500 µg/mL of pHrodo™ Red Zymosan BioParticles ('Zymosan', Life Technologies, USA) were vortexed and resuspended homogeneously in RPMI (pH = 7.4). Cultures were rinsed off in PBS three times to remove extracellular parasites before activation. Each well was supplemented with dispersed 'Zymosan' 200 µL/well at 2, 6, 12, and 24 hpi, and incubated at 37 ˚C without CO_2 supplementation for further 2 h after induction of activation,. Infections were repeated 6 times per time point for each culture. The reaction was washed 3 times with PBS. Then ice-cold PBS was added to stop the reaction for cell imaging. NucBlue™ Live ReadyProbes™ (Life Technoglogies, USA) were used to stain nuclei. In order to test for basic function of phagocytosis by cells treated according to the described conditions, cultures of group NC (n = 6 per observation period) were exposed to activation at 2, 6, 12 and 24 hpi (infection time point for other infection groups). Additionally, uninfected control cultures (n = 3) were stimulated for 1 h by LPS application 96 h after seeding. Non-cell controls were kept in parallel. All incubation steps were performed in a dark chamber.

Confocal laser scanning microscopy (CLSM)

Phagocytosis was determined by cell imaging with 200x magnification using CLSM (Leica TCS SP8, Wetzlar, Germany). Imaging spots were taken from the central area of six individual wells for each group and observation period. Parasite visualization (400 x magnifications) was carried out 12 hpi. Stacks were calculated for every 1.5-2 µm and included all attached cells. Imaris® software version 9.3 (Bitplane, Abingdon, UK) was used to generate a 3D model from the stacks and to quantify the number of intracellular granules of 'Zymosan' per cell. The relative inhibition of phagocytosis in infected cultures following 2 h of stimulation by 'Zymosan' was calculated as follows:

$$Relative\ Inhibition = 1 - \frac{number\ of\ 'Zymosan'\ positive\ cells\ in\ infected\ group}{number\ of\ 'Zymosan'\ positive\ cells\ in\ non-infected\ control}$$

Intracellular parasite replication

Cultures were trypsinized by Biotase® (Biochrom, Berlin, Germany) at 37 ˚C for 30 min and collected at 2, 6, 12, and 24 hpi after washing three times gently with PBS. DNA was extracted using the QIAamp DNA Mini Kit® (Qiagen, Hilden, Germany) following the manufacturer's instructions for cell cultures. *T. gondii* DNA standard curve was obtained by gradient 10-fold dilutions of 10^7 tachyzoites. *T. gondii* replication was analyzed by the 529-bp repeat element in a probe-based qPCR. ITS1 fragment quantification was used to quantify the replication of *E. tenella* by SYBR Green-based PCR (KAWAHARA, et al. 2008).

According to the chosen infection doses, DNA was extracted from 2.5 x 10^5 and 5 x 10^5 *E. tenella* sporozoites, respectively, to normalize the initial copy numbers of *E. tenella* (n=4). The relative copy number of *E. tenella* DNA was implemented by measurement of pSCA-17 plasmid standard dilution as described before (THABET, et al. 2015). Quantitative real-time PCR (qPCR) was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, USA).

For *T. gondii* qPCR, 5 µL of sample DNA eluate were used in a total volume of 25 µL containing 12.5 µL of Master Mix, 3.2 µL of DNase/RNase free water (Gibco™, Life Technologies, USA), 0.9 µL of each 25 µM forward and reverse primer and 2.5 µL of 2 µM TaqMan probe. The cycling

program consisted of 95 ℃ for 15 min (initial denaturation), followed by 40 cycles of 95 ℃ for 15 s (denaturation), 60 ℃ for 1 min (annealing), and 72 ℃ for 15 s (extension). 2 µL of sample DNA eluate were used for *E. tenella* specific PCR, in a total volume of 20 µL containing 10 µL of SYBR Green master mix, 7.2 µL of water, 0.9 µL of 25 µM forward and reverse primer. For *E. tenella* qPCR, the cycling program consisted of heating to 95 ℃ for 5 min (initial denaturation), followed by 40 cycles at 95 ℃ for 30 s (denaturation), 55 ℃ for 20 s (annealing), and 72 ℃ for 20 s (extension). A subsequent melting curve analysis (95 ℃ for 1 min, 55 ℃ for 30 s, 0.5 ℃/s) was performed for *E. tenella* qPCR to create the dissociation curve. Data represent the mean of three replicates with an acceptable standard deviation of less than 0.5 for Ct values.

Data analysis and statistics

The intracellular *T. gondii* tachyzoites were represented as total DNA copy quantities. For *E. tenella* the DNA copy number/µL was assessed for each observation period. RPN was calculated as mean value (n = 3) with standard deviation (SD) for each group in relation to DNA copy numbers determined for the initial infection dose. Statistical analysis was calculated by SPSS® version 20 (IBM, New York, USA). Differences between groups were determined by the nonparametric Mann-Whitney U test and assumed significant at $p < 0.05$.

List of abbreviations

BMDMs: bone marrow-derived macrophages; CLSM: Confocal laser scanning microscopy; hpi: hours post infection; HFF: human foreskin fibroblast; iNOS: nitric oxide synthase; LPS: Lipopolysaccharide; MOI: multiplicity of infection; PBS: phosphate-buffered saline; PMBC: peripheral blood mononuclear cells; PV: parasitophorous vacuole; qPCR: Quantitative real-time PCR; RPN: Relative parasite numbers; SD: standard deviation.

Ethics approval and consent to participate

The animal experiments related to the blood sampling were approved by the responsible authorities (Landesdirektion Sachsen, Germany, trial registration number V13/10)

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests

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Authors' contribution

RZ designed the study, wrote the manuscript, performed the experiments and analysed partial data. WZ analysed partial data and helped to draft the manuscript. AD and BB critically revised the study design, data interpretation and manuscript. All authors read and approved the final manuscript.

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2.2.4 Manuscript 4: Monocyte-derived chicken macrophages exposed to *Eimeria tenella* sporozoites display reduced susceptibility to invasion by *Toxoplasma gondii* tachyzoites.

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I. Concept

Runhui Zhang was responsible for the idea and design of this work with the critical evolution and inspection of research goal and aims by Berit Bangoura and Arwid Daugschies.

II. Investigation

Runhui Zhang conducted the research and investigation process including performing the experiments with the participation of Wanpeng Zheng (data collection)

III. Analysis

Runhui Zhang performed data analysis.

IV. Manuscript

Runhui Zhang wrote the initial draft and prepared the published work with critical revision and writing improvement by Wanpeng Zheng, Berit Bangoura and Arwid Daugschies.

Monocyte-derived chicken macrophages exposed to *Eimeria tenella* sporozoites display reduced susceptibility to invasion by *Toxoplasma gondii* tachyzoites

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Abstract

Both *Eimeria (E.) tenella* and *Toxoplasma (T.) gondii* are common apicomplexan parasites in chickens. Host cell invasion by both protozoans includes gliding motility, host cell attachment and active penetration. Chicken macrophages as phagocytic cells participate in the innate host immune response against these two parasites. In this study, primary chicken monocyte-derived macrophages (MMs) were infected with both pathogens to investigate mutual and host-parasite interactions. MMs cultures were assigned to groups that were infected with *E. tenella*, *T. gondii*, or both. In co-infected cultures, MMs were first exposed to *E. tenella* sporozoites for 2 hours. Afterwards, *T. gondii* tachyzoite infection was performed. Live-cell imaging was carried out to observe cell invasion and survival of *T. gondii* by single parasite tracking over a period of 20 hours post infection (hpi). Quantitative analysis for parasite replication was performed at 2, 6, 12 and 24 hpi by real-time PCR (qPCR). Overall, we found that during early co-infection *T. gondii* tachyzoites adhered for more than 4 hours to macrophages. Although they displayed high motility, ability to penetrate the cell

membrane of the potential host cell was reduced. qPCR results confirmed that significantly less *T. gondii* entered *E. tenella*-activated MMs at 2 hpi, and a reduced proportion of intracellular *T. gondii* survived and replicated in these cells at 24 hpi. We conclude that *E. tenella* modulates host cell responses to another apicomplexan agent, *T. gondii*, reducing active invasion and multiplication in chicken primary macrophages.

1 Introduction

Apicomplexan protozoa are obligate intracellular parasites causing a variety of diseases in animals and humans. *Toxoplasma (T.) gondii* infects almost all euthermic animal species and may also infect humans. About one third of the global human population are supposed to be infected by this zoonotic pathogen (Hill and Dubey, 2002). Chickens are considered to be an important reservoir of *T. gondii*. Especially free-ranging and back-yard chickens have been reported to show high seroprevalence rates for *T. gondii* (Dubey, 2010). On the other hand, coccidiosis caused by *Eimeria* species is a widely distributed major parasitic disease of poultry, one of the most pathogenic species in chickens being *E. tenella*.

Upon adherence to the host cell membrane apicomplexan parasites start to penetrate into the host cell by forming a so-called moving junction initiated by organelles of the apical complex at the anterior end of the parasites (Besteiro et al., 2011). This moving junction supports the establishment of the parasitophorous vacuole (PV) separating the parasite from the host cell cytoplasma. The adhesive proteins produced by micronemes located in the apical region of the invasive parasite stages allow gliding motility which is essential for the active invasion by *T. gondii* (Sibley, 2010). In chickens, macrophages play a crucial role in identification and phagocytosis of pathogens including protozoa thus serving as a first line of innate immune defence (Qureshi et al., 2000). However, *T. gondii* tachyzoites are capable to actively invade macrophages which occurs even faster than phagocytosis displayed by a macrophage (Morisaki et al., 1995). *T. gondii* tachyzoites are able to replicate in chicken blood monocyte-derived macrophages (Meirelles and Souza, 1985). In experimental infection of *E. tenella* in naïve chicken, sporozoites were situated mainly within or next to the lamina propria that was infiltrated with macrophages in response to the infection (Vervelde, et al., 1996). Sporozoite-bearing macrophages are able to transport *Eimeria* sporozoites to the proper site of the intestinal mucosa (Trout and Lillehoi, 1993). However, *E. tenella* survival and development is rather poor in cultured chicken macrophages (Challey and Burns, 1959).

Concomitant infections by protozoan parasites and other microoganisms attract increasing attention in animals and humans both under natural conditions and in experimental *in vivo* and *in vitro* studies. For instance, probiotic bacteria reduced oocyst excretion of *E. acervulina* in chicken (Dalloul et al.,

2003) as well as *in vitro* invasion of *E. tenella* into Madin-Darby bovine kidney (MDBK) cells (Tierney et al., 2004). Host immune responses and consequently clinical signs of disease are modulated in co-infected animals. In general, the immune response is dominated by Th1 during protozoan infection whereas Th2 response is typical for helminth infection (Marshall et al., 1999). Regarding interaction during co-infection by protozoa, it was reported that no competitive effects exist in mixed *Eimeria* species infection (Cornelissen et al., 2009). However, *T. gondii* replication was increased during co-infection with *Trypanosoma* in rats (Guerrero et al., 1997). On the other hand, *T. gondii* supports *Plasmodium* replication in a rat model (Rifaat et al., 1984). Natural co-occurrence of *T. gondii* and *Eimeria* in the same host has been reported. For example, both parasites were found concurrently in blood and organs of a heavily diseased sparrow (Manwell et al., 1945). Furthermore, wild rabbits tested seropositive for both *T. gondii* and *Eimeria stiedai* infection in Scotland (Mason et al., 2015).

In spite of high seroprevalence rates reported for both *T. gondii* and *E. tenella* in chickens, little is known to date about the mutual interplay between these two parasites, particularly during host cell invasion. Experimental *in vivo* and *ex vivo* co-infection models in chicken macrophages were established recently and thus suitable tools are now available for further co-infection studies (Hiob et al., 2017; Zhang et al., 2018). Results demonstrated that the mutual interaction during co-infection modulated both parasite replication as well as the host immune response. In our recent study, macrophage phagocytosis was distinctly altered during *in vitro* co-infection by *T. gondii* and *E. tenella* (unpublished yet).

In the current study, we used live-cell imaging of *T. gondii* and/or *E. tenella* infected cells to monitor parasite invasion and survival at the single cell-parasite level in both mono-infected and co-infected cultures. The study focuses on the period of early parasite invasion in monocyte-derived chicken macrophages. A previous study showed that asexual stages of *T. gondii* and *Eimeria* displayed ultrastructural similarities (Soares Medeiros et al., 2011). Moreover, host cell invasion mechanisms, micronemal adhesins, gliding motility of both *T. gondii* tachyzoites and *Eimeria* sporozoites were identified to be affected *in vitro* by the same inhibitor of protozoan cGMP-dependent protein kinase (Wiersma et al., 2004). Thus, we are particularly interested in whether the considerable capacity of *T. gondii* to invade and survive in macrophages is affected by concorrent *E. tenella* infection. Parasite tracking was performed to determine the motility of *T. gondii* during the invasion phase following co-infection by *E. tenella*. The life span of individual *T. gondii* was also monitored by live cell imaging.

2 Materials and Methods

2.1 Parasites and host cells

Tachyzoites of *T. gondii* RH - green fluorescent protein (GFP) strain (kindly provided by Professor Dominique Soldati-Favre, University of Geneva Medical School, Switzerland) were maintained at 37 ℃ with 5% CO_2 in human foreskin fibroblast (HFF) cells. Free tachyzoites were collected from the culture medium for infection. The sporozoites of *E. tenella* Houghton - yellow fluorescent protein (YFP) strain (kindly provided by Prof. Dr. Suo, China Agricultural University, China) were obtained from oocysts by excystation following an established protocol (Renter á-Sol ś et al., 2020).

The animal experiments performed to collect chicken blood samples were approved by the responsible authorities (Landesdirektion Sachsen, Germany, trial registration number V13/10). Chicken peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood of adult chickens according to the established protocols with slight modifications kindly provided by Dr. Braukmann, Friedrich-Loeffler-Institute Jena, Germany. Briefly, PBMCs were separated from blood by centrifugation (250 x *g*, 45 min) with Biocoll (density 1.077 g/ml; Biochrom AG, Berlin, Germany). Isolated PBMCs were resuspended and washed in 5 mL PBS (centrifugation 350 x *g*, 30 min) following pre-warmed 5 mL RPMI-1640 medium (Sigma, Taufkirchen, Germany) (centrifugation 350 x *g*, 20 min). Afterwards, 5 x 10^6 PBMCs/well were resuspended in 24-well-plates in RPMI-1640 with 5% chicken serum and 5% fetal bovine serum, penicillin (100 U/mL, PAA), streptomycin (0.1 mg/mL, PAA), and amphotericin B (0.0025 mg/mL, PAA). After 72 h incubation (41 ℃, 5 % CO_2) PMBCs were trypsinized by Biotase® (Biochrom, Berlin, Germany) at 37 ˚C for 30 min and detached monocyte derived macrophages (MMs) were counted under the light microscope. 10^3 MMs were seeded in the microscope imaging chamber (micro-insert 4 well, Ibidi, Martinsried, Germany) for 24 h (41 ℃, 5 % CO_2) for live cell imaging. For parasite quantification by qPCR, approximately 10^5 MMs were seeded to 24-well-plates for 24 h (41 ℃, 5 % CO_2). The MMs were purified by rinsing off non-adherent cells once at 24 h and twice before infection.

2.2 Infection

Two experiments were conducted in this study (Fig 1): Experiment 1 was designed to visualize parasite invasion; Experiment 2 was performed with increased MMs population at the same infection ratio to quantify parasite stages by qPCR for the different infection groups.

Study design for experiment 1 (Fig 1A):

For imaging, cell cultures (n = 2/group) were assigned to four groups. In co-infected group CI, MMs were exposed to 2 x 10^3 *E. tenella* sporozoites 2 h before *T. gondii* infection (-2 hpi). Group LPS cultures served as positive controls and were stimulated at the same time with 1 µg/mL lipopolysaccharide (LPS). Cultures of group Tg (monoinfection with *T. gondii*) and group NC (negative control) were not exposed to LPS stimulation. At 0 hpi cultures of groups CI, LPS, and Tg were infected with 2 x 10^3 *T. gondii* tachyzoites per well. Group NC remained uninfected. All cultures were observed until 20 hpi. The whole experiment was repeated once.

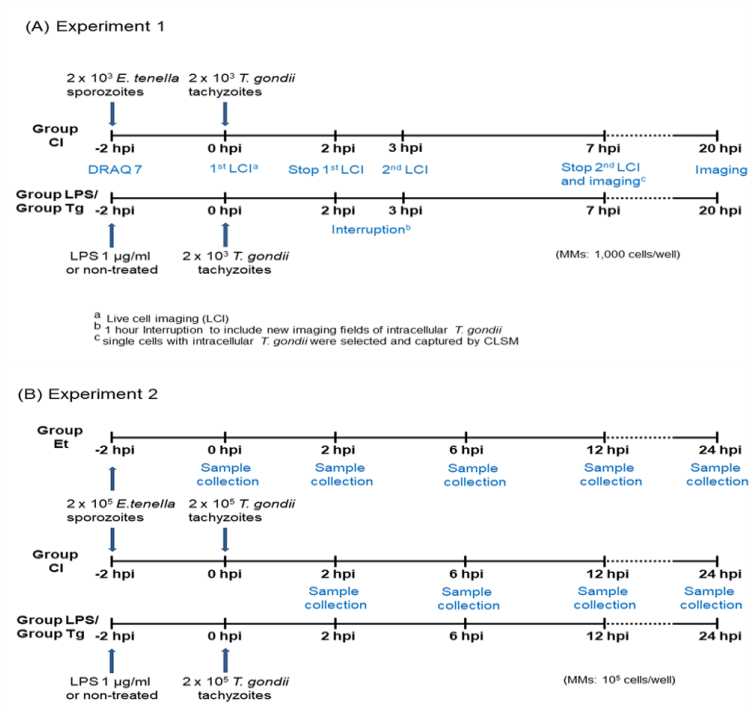
Study design for experiment 2 (Fig 1B):

For quantification by qPCR, cell cultures kept in 24-well plates were assigned to five groups (n=5 cultures/group). At -2 hpi, MMs of group CI (co-infection) and group Et (single infection with *E. tenella*) were exposed to 2 x 10^5 *E. tenella* sporozoites per culture. Group LPS cultures were pretreated with 1 µg/ml LPS at the same time. Group Tg was infected with 2 x 10^5 *T. gondii* tachyzoites per well at 0 hpi while group NC served as untreated, uninfected negative control. The cultures were maintained until 24 hpi.

Figure 1 Schematic representation of infection and imaging.

(A) Experiment 1 (live cell imaging). Images of group NC were collected at the same time points as in groups CI, LPS and Tg.

(B) Experiment 2 (parasite quantification by qPCR). Samples of group NC were collected at the same time point as in groups CI, LPS and Tg.



2.3 Live-cell imaging of *T. gondii* in MMs

DRAQ7 dye was used to assess the viability of parasites and macrophages in each group. Prior to imaging, 3 µL DRAQ7 dye (Biostatus, Leicestershire, UK), a nuclear stain selective for dead cells, was added to all cell cultures immediately after exposure to *E. tenella* or LPS treatment (-2 hpi). The viability of parasites and cells were controlled by fluorescent microscopy prior to *T. gondii* infection at 0 hpi. Cells were viewed for fluorescence by CLSM (TCS-SP8, Leica, Bensheim, Germany) using 4 channels at 488 nm, 514 nm, 633 nm laser line and wide field. Basic imaging parameters were 40 x objective, 10 x ocular (NA 0.90), 1024 x 1024 dpi, 6 Z-stacks (4 µm). To avoid crosstalk between channels, images were collected in line sequential mode.

Incubation conditions (41 °C, 5 % CO_2 and 99% humidity) were controlled using an incubation chamber (Tokai-Hit, Shizuoka, Japan) over the whole observation period. The timeframe of infection and imaging is shown in Fig. 1A. Briefly, after adding DRAQ7, approximately 20 random fields of cells were selected and captured according to groups CI, LPS, and Tg from 2 individual wells per group. Image collection for time-lapse imaging of tachyzoite motility was performed at an interval of 10 min per frame until 2 hpi. Image acquisition was interrupted from 2 hpi to 3 hpi to add 20 additional fields per group which contained intracellular *T. gondii* in group CI and LPS. All fields were observed for further 4 h until 7 hpi at an interval of 30 min per frame. Subsequently, at least 100 cells with live intracellular *T. gondii* (1 - 2 tachyzoites / cell) were collected and captured at 7 hpi and 20 hpi by CLSM for groups CI and Tg. For group LPS, only 71 MMs with live intracellular *T. gondii* were selected due to a generally low number of *T. gondii*-positive cells observed in this group. Six fields were analysed randomly for group NC in parallel.

Images of stacks were obtained using LAS X software (Leica, Bensheim, Germany). Stacks were analysed with Imaris® software version 9.3 (Bitplane, Abingdon, UK) using functions of spot detection and tracking parasite motility and viability.

2.4 Parasite quantification by quantitative real-time PCR (qPCR)

For all infection groups (Fig 1B), complete cell populations from a subset of cell culture wells were collected at 2, 6, 12, and 24 hpi and additionally at 0 hpi in group Et. DNA was extracted using the QIAamp DNA Mini Kit® (Qiagen, Hilden, Germany) following the manufacturer's instructions. qPCR was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, USA). Data represent the mean of three replicates with an acceptable standard deviation of less than 0.5 for Ct values.

T. gondii multiplication was analyzed by a probe-based qPCR detecting the 529-bp repeat element (Edvinsson et al., 2006). Standard curve was developed by data obtained for gradient 10-fold dilutions of initially 10^7 tachyzoites. qPCR was conducted in a total volume of 25 µL: 5 µL of sample DNA, 12.5 µL of Master Mix, 3.2 µL of DNase/RNase free water (Gibco™, Life Technologies, USA), 2.5 µL of 2 µM TaqMan probe and 0.9 µL of each 25 µM forward and reverse primer (5'-CACAGAAGGGACAGAAGT and 5'-TCGCCTTCATCTACAGTC-3'). The cycling program consisted of 95 ℃ for 15 min, followed by 40 cycles of 95 ℃ for 15 s, 60 ℃ for 1 min, and 72 ℃ for 15 s.

ITS1 fragment quantification was used to assess the replication of *E. tenella* by a SYBR Green-based PCR (Kawahara et al., 2008). The relative copy number of *E. tenella* DNA was implemented by measurement of pSCA-17 plasmid standard dilution as described before (Thabet et al., 2015). qPCR was conducted in a total volume of 20 µL: 2 µL of sample DNA eluate, 10 µL of SYBR Green master mix (Thermo Fisher Scientific, Darmstadt, Germany), 7.2 µL of water, 0.9 µL of 25 µM forward and reverse primer (5'-AACCTGACTGTGCAAGCATC-3' and 5'-ATCATAGACAGCCGTGCCAG- 3'). The cycling program consisted of heating to 95 ℃ for 5 min, followed by 40 cycles at 95 ℃ for 30 s, 55 ℃ for 20 s, and 72 ℃ for 20 s. A subsequent melting curve analysis (95 ℃ for 1 min, 55 ℃ for 30 s, 0.5 ℃/s) was performed to create the dissociation curve.

2.5 Statistical analysis

Statistical analysis was performed by using Imaris® and GraphPad Prism® (version 8, San Diego, CA, USA) software. Kolmogorov-Smirnov test was performed to test for normal distribution. Statistical significance was assessed by two-way ANOVA for data with normal distribution and Tukey's multiple comparisons test for values that did not follow normal distribution.

3 Results

3.1 Live cell imaging of *T. gondii* in MMs

In general, penetration of *T. gondii* tachyzoites into the MMs started within 2 hpi after tachyzoites were seeded into cultures. CLSM analysis (Fig 2A and Supplementary material: Video 1) of group CI cultures showed that *T. gondii* tachyzoites remained loosely adherent to MMs for more than 4 hours in most cases before they started to actively invade the host cell. During this phase, distinct helical gliding motility of tachyzoites was seen. This untypical behavior of tachyzoites in group CI was observed regardless whether intracellular *E. tenella* sporozoites were still alive or dead. In

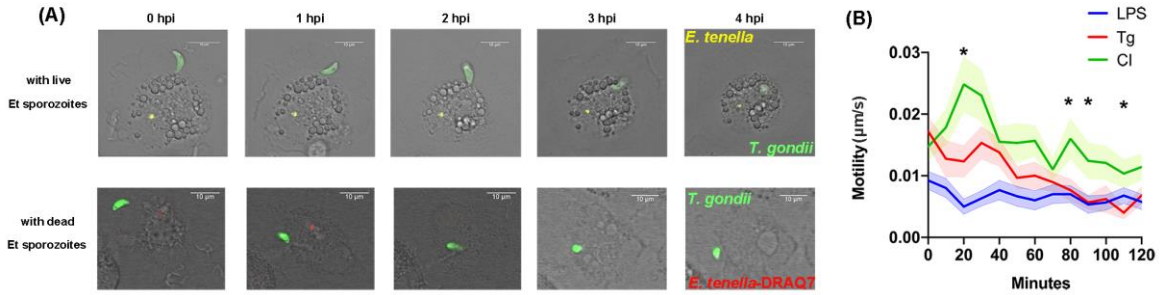
contrast, tachyzoites, which were actively or passively incorporated into MMs after attachment, were seen within minutes in the mono-infected group Tg and the LPS-treated group

Tachyzoites showed lowest motility in group LPS at most of the captured time points (Fig 2B). In group CI, significantly higher motility of tachyzoites were observed than in group LPS over the period from 10 min to 120 min post infection ($p < 0.05$); with the exception of 70 to 110 min. Comparing between groups CI and Tg, tachyzoites of group CI showed significantly higher motility ($p < 0.05$) during the first 20 mins. Average motility values in group CI tended to remain higher than in both groups Tg and LPS with a significant difference ($p < 0.05$) at 80, 90 and 110 min compared to group Tg.

Figure 2 Invasion of *T. gondii* within 4 hpi.

(A) Video microscopy of *T. gondii* invasion at 0-4 hpi in a cell previously exposed to *E. tenella*. (a) *T. gondii* is adherent on the cell which contains a vital *E. tenella* sporozoite. (b) *T. gondii* is adherent on the cell which contains a dead *E. tenella* sporozoite that was phagocytized and fused by macrophages at 2 hpi.

(B) Motility (speed) of live *T. gondii* tachyzoites (0-120 minutes). The motility of *T. gondii* tachyzoites (n=30 per group) was assessed by imaging every 10 minutes over 120 minutes. Each parasite was tracked over 70-100% of all time points excluding the occasionally out-of-range movement or parasite death. Mean values per time point were calculated. LPS: LPS-treated, *T. gondii* mono-infection; Tg: *T. gondii* mono-infection; CI: co-infection. *p < 0.05: CI compared to Tg; Error bar: standard error of the mean (SEM)



3.2 Parasite quantification

In experiment 1, the relative intensity (RI) of green fluorescence of 27 to 30 individual intracellular *T. gondii* tachyzoites which were captured at 3 to 7 hpi by CLSM were anlaysed in all infected groups. RI revealed no significant difference between groups Tg and CI until 4 hpi. Thereafter, RI values

remained on a consistently higher level in group CI than in the monoinfected group Tg and in group LPS (Fig 3A).The RI measured in group CI displayed only slight variation from 3 to 7 hpi, whereas values started to decrease in group LPS and Tg following 4.5 hpi or 5 hpi with a statistically significant difference between groups CI and LPS ($p < 0.05$) at 4.5 hpi and thereafter.

In general, less than 45 % of traced intracellular tachyzoites of *T. gondii* were still alive or replicating in MMs at 20 hpi (Fig 3B). At 20 hpi, the proportion of *T. gondii*-positive MMs was lower in group CI (27 %) than in group Tg (45%) and only slightly higher than in group LPS (21 %).

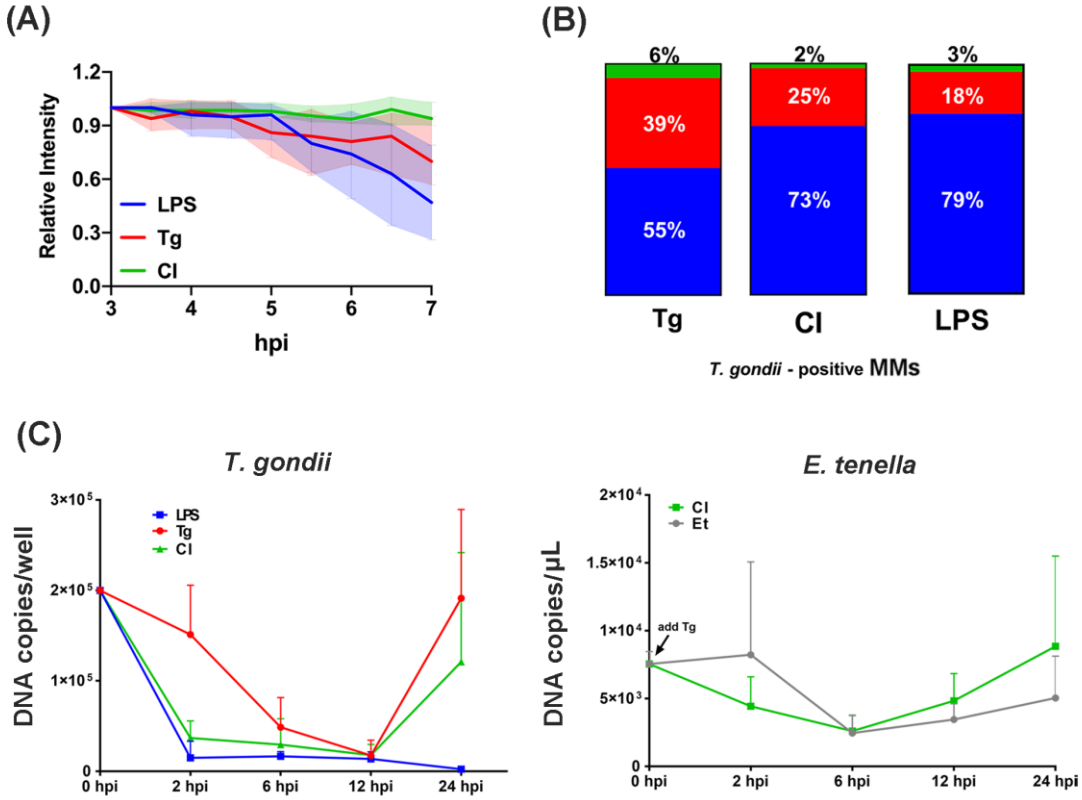
By qPCR, significantly lower DNA copy numbers ($p < 0.05$) were determined at 2 hpi for *T. gondii* in group CI and group LPS compared to group Tg (Fig 3C, left). The number of DNA copies remained on a low level in the two former groups until 12 hpi, followed by a steep increase until 24 hpi in group CI while values remained significantly lower in group LPS ($p < 0.05$). In group Tg, DNA copy numbers were higher than in the two other groups at 2 hpi, decreased steadily until 12 hpi to a level similar to groups CI and LPS and increased even more distinct than those recorded for group CI thereafter. No statistical difference was observed between group CI and Tg at this time point ($p > 0.05$). Likewise, no significant difference in DNA copy numbers was detected for *E. tenella* by comparison of groups Et and CI over the entire observation period of 24 hpi (Fig. 3C, right).

Figure 3 Quantitative analysis of *T. gondii*

(A) Assessment of intracellular survival of *T. gondii* (n=27-30) by measurement of Relative Intensity (RI) at 3-7 hpi.. RI = initial mean intensity of fluorescence for each parasite / mean intensity of fluorescence for each parasite per time point. RI = 0 means no signal of fluorescence is detected. Error bar: standard error of the mean.

(B) *T. gondii* negative/positive MMs. 100 vital *T. gondii*-positive MMs in group CI and Tg and 71 MMs in group LPS (due to low numbers of surviving parasites) were selected and marked randomly, i.e. 1-2 vital intracellular tachyzoites / cell were captured at 7 hpi and again at 20 hpi. The rate of *T. gondii*-negative/positive cells was calculated. Blue column, MMs with reduced numbers of or non-vital *T. gondii*; Red column, MMs with constant numbers of vital *T. gondii*; Green column, MMs with replicating *T. gondii*.

(C) Parasite quantities (DNA copies) during sequential co-infection. Parasite replication is represented as mean value with standard deviation (n=3-5 per time point). LPS: LPS-treatment, *T. gondii* mono-infection; Tg: *T. gondii* mono-infection; Et: *E. tenella* mono-infection; CI: co-infection



4 Discussion

After two hours of exposure to *E. tenella* sporozoites or LPS MMs generally maintained their viability which was demonstrated by negative staining for DRAQ7 (data not shown). A recent study by (Zhang et al., 2018), confirmed earlier findings by (Long and Rose, 1976) that *E. tenella* sporozoites were mostly located within chicken macrophages at 2 hpi. In the current study, most intracellular *E. tenella* sporozoites showed red fluorescescence after DRAQ7 staining instead of the expected yellow (YFP) fluorescence within 4 hpi to 6 hpi (data not shown), indicating their death. This CLSM finding corroborates the qPCR results demonstrate that the number of *E. tenella* declined to lowest numbers at 6 hpi (Fig 3A, right). Co-infection with *T. gondii* did not exhibit a significant influence on *E. tenella* replication when compared to single *E. tenella* infection. Unfortunately, we could not quantify the *E. tenella* sporozoite numbers via YFP through time lapsing imaging because of a non-ideal YFP expression (about 80 %) in the available parasite strain.

Host cell invasion by *T. gondii* tachyzoites usually takes only 15 to 20 s (Morisaki et al.. It is assumed that *T. gondii* tachyzoites and *Eimeria* sporozoites may invade and traverse several host cells by disrupting the host cell membrane (Mota and Rodriguez, 2001). In our time lapsing study, *T. gondii* tachyzoites were either floating free in the culture medium (unattached to macrophages) for

hours or displayed a rapid entry into the macrophages in both group LPS and group Tg. Interestingly, adherence of vital tachyzoites was prolonged for more than 4 hours in the co-infection cultures. It was previously demonstrated that most *T. gondii* tachyzoites remained adherent to murine macrophages that were treated with a phagocytosis inhibitor, Cytochalasin D (Ryning and Remington, 1978). Microneme exocytosis is necessary for host-cell entry of both *T. gondii* (Carruthers et al., 1999) and *E. tenella* (Bumstead and Tomley, 2000). The micronemes appear to be both structurally and functionally conserved between different members of the Apicomplexa (Sibley, 2010). We assumed that *E. tenella* infection may potentially hamper the entry of *T. gondii* into host cells by altering recognition of signal receptors or inhibiting phagocytosis.

Rapid invasion and egress are crucial to *T. gondii* survival and successful replication, thereby minimizing the exposure to destructive reaction by innate protection in a generally hostile extracellular environment (Hoff and Carruthers, 2002). Once inside the host cell, the parasite no longer moves (Dubremetz et al., 1998). The intracellular survival of *T. gondii* depends on the route by which the parasite enters the host cell. Although *T. gondii* tachyzoites are phagocytized and internalized through Fc receptor mediation (Mordue and Sibley, 1997), intracellular *T. gondii* can survive and replicate within the PV by blocking the host macrophage's pathways intended to initiate vacuolar acidification and parasite inactivation (Sibley et al, 1985). To quantify viable intracellular *T. gondii*, the intensity of GFP signal expression by the parasite was monitored in this study. It appeared from our observations according to RI values that *T. gondii* showed better survival at the early stage of infection (until 7 hpi) if MMs were previously exposed to *E. tenella* (Fig 3A).

Under the conditions of our experimental design (two *T. gondii* tachyzoites per cell), most infected host cells contained only one parasite at 7 hpi in all infected groups. Only slightly more *T. gondii*-positive cells were monitored in group CI than in group LPS at 20 hpi by CLSM (Fig. 3B). However, significant high number of total DNA copies of *T. gondii* was detected in group CI compared to group LPS (Fig. 3C). It appears that the single cell observation by CLSM may not be completely comparable with quantification of DNA copies in cell cultures, possibly due to the different amount of cells considered. Nonetheless, results obtained with both methods indicated a reduced growth of *T. gondii* in chicken macrophages in a co-infection setting with *E. tenella* compared to mono-infection.

Macrophages are not only professional phagocytes but also secrete cytokines in response to parasite infection. This innate immune response can be triggered in chicken macrophages by exposure to LPS (Hussain and Qureshi, 1998). A previous study showed that *T. gondii* blocked LPS-induced production of IL-12 and TNF-alpha in murine bone marrow derived macrophages (Butcher and

Denkers, 2002). For *E. tenella*, it is known that IL-1β and iNOS expression are significantly enhanced in chicken HTC macrophages by merozoites at 2 hpi (Chow et al., 2011). A recent *in vitro* study demonstrated down-regulation of IL-12 and iNOS in chicken macrophages during simultaneous co-infection by *T. gondii* and *E. tenella* (Zhang et al., 2018). From previous as well as the current data it appears that modulation of innate immunity in chicken differs during mono- and co-infection, which includes cytokine production and macrophage phagocytosis. However, data on concurrent infections with *E. tenella* and *T. gondii* are still scarce although both are considered to be common pathogens in poultry and thus deserve more attention.

In summary, life cell imaging by CLSM proved to be a useful tool to evaluate chicken macrophage invasion and / or phagocytosis during mono- and co-infection with two different apicomplexan parasites. It was demonstrated that the mechanisms of *T. gondii* invasion and survival appear to be altered in *E. tenella*-exposed macrophages. Further studies into macrophage signaling pathways, particularly modulation of the cytokine response, combined with image analysis and live cell imaging, will help to better understand the function and modulation of the innate immune response during apicomplexan invasion.

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6 Author Contributions Statement

RZ designed the study, wrote the manuscript, and performed the experiments and data analysis. WZ helped to perform cell imaging and manuscript drafting. AD and BB critically revised the study design, data interpretation and manuscript. All authors read and approved the final manuscript.

7 Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8 Ethics Statement

The animal experiments performed to collect chicken blood samples were approved by the responsible authorities (Landesdirektion Sachsen, Germany, trial registration number V13/10)

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10 References

Besteiro, S., Dubremetz, J. F., and Lebrun, M. (2011). The moving junction of apicomplexan parasites: a key structure for invasion. *Cell Microbiol*.13, 797–805. doi: 10.1111/j.1462-5822.2011.01597.x

Bumstead, J., and Tomley, F. (2000). Induction of secretion and surface capping of microneme proteins in *Eimeria tenella*. *Mol Biochem Parasitol.* 110, 311–321. doi: 10.1016/s0166-6851(00)00280-2

Butcher, B. A., and Denkers, E. Y. (2002). Mechanism of entry determines the ability of *Toxoplasma gondii* to inhibit macrophage proinflammatory cytokine production. *Infect Immun.* 70, 5216–5224. doi: 10.1128/iai.70.9.5216-5224.2002

Carruthers, V. B., Giddings, O. K., and Sibley, L. D. (1999). Secretion of micronemal proteins is associated with *Toxoplasma* invasion of host cells. *Cell Microbiol.* 1, 225–235.

Challey, J. R., and Burns, W. C. (1959). The invasion of the cecal mucosa by *Eimeria tenella* sporozoites and their transport by macrophages. *J Protozool* 6, 238–241. doi: j.1550-7408.1959.tb04364.x

Chow, Y.-P., Wan, K.-L., Blake, D. P., Tomley, F., and Nathan, S. (2011). Immunogenic *Eimeria tenella* glycosylphosphatidylinositol-anchored surface antigens (SAGs) induce inflammatory responses in avian macrophages. *PloS One* 6: e25233. doi: 10.1371/journal.pone.0025233

Cornelissen, J.B.W.J., Swinkels, W.J.C., Boersma, W. A., and Rebel, J.M.J. (2009). Host response to simultaneous infections with *Eimeria acervulina*, *maxima* and *tenella*. A cumulation of single responses. *Vet Parasitol.* 162, 58–66. doi: 10.1016/j.vetpar.2009.02.001

Dalloul, R. A., Lillehoj, H. S., Shellem, T. A., and Doerr, J. A. (2003). Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a Lactobacillus-based probiotic. *Poult Sci.* 82, 62–66. doi:10.1093/ps/82.1.62

Dubey, J. P. (2010). *Toxoplasma gondii* infections in chickens (Gallus domesticus). Prevalence,

clinical disease, diagnosis and public health significance. *Zoonoses Public Health.* 57, 60–73. doi: 10.1111/j.1863-2378.2009.01274.x

Dubremetz, J. F., Garcia-Réguet, N., Conseil, V., and Fourmaux, M. N. (1998). Invited review Apical organelles and host-cell invasion by Apicomplexa. *Int J Parasitol.* 28, 1007–1013. doi: 10.1016/s0020-7519(98)00076-9

Edvinsson, B., Lappalainen, M., and Evengård, B. (2006). Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis. *Clin Microbiol Infect.* 12, 131–136. doi: 10.1111/j.1469-0691.2005.01332.x

Guerrero, O. M., Chinchilla, M., and Abrahams, E. (1997). Increasing of *Toxoplasma gondii* (*Coccidia*, *Sarcocystidae*) infections by *Trypanosoma lewisi* (*Kinetoplastida*, *Trypanosomatidae*) in white rats. *Rev Biol Trop.* 45, 877–882.

Hill, D., and Dubey, J. P. (2002). *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin Microbiol Infect.* 8, 634–640. doi: 10.1046/j.1469-0691.2002.00485.x

Hiob, L., Koethe, M., Schares, G., Goroll, T., Daugschies, A., and Bangoura, B. (2017). Experimental *Toxoplasma gondii* and *Eimeria tenella* co-infection in chickens. *Parasitol Res.* 116, 3189-3203. doi: 10.1007/s00436-017-5636-2

Hoff, E. F., and Carruthers, V. B. (2002). Is *Toxoplasma* egress the first step in invasion? *Trends Parasitol.* 18, 251–255. doi: 10.1016/s1471-4922(02)02240-7

Hussain, I., and Qureshi, M. A. (1998). The expression and regulation of inducible nitric oxide synthase gene differ in macrophages from chickens of different genetic background. *Vet Immunol Immunopathol.* 61, 317–329. doi: 10.1016/s0165-2427(97)00153-0

Kawahara, F., Taira, K., Nagai, S., Onaga, H., Onuma, M., and Nunoya, T. (2008). Detection of five avian *Eimeria* species by species-specific real-time polymerase chain reaction assay. *Avian Dis.* 52, 652–656.doi: 10.1637/8351-050908-Reg.1

Long, P. L., and Rose, M. E. (1976). Growth of *Eimeria tenella in vitro* in macrophages from chicken peritoneal exudates. *Z. Parasitenkd.* 48, 291-294. doi: 10.1007/BF00380405.

Manwell, R. D., Coulston, F., Binckley, E. C., and Jones, V. P. (1945). Mammalian and avian *Toxoplasma*. *J Infect Dis.* 76, 1–14.

Marshall, A. J., Brunet, L. R., van Gessel, Y., Alcaraz, A., Bliss, S. K., Pearce, E. J., and Denkers, E.

Y. (1999). *Toxoplasma gondii* and *Schistosoma mansoni* synergize to promote hepatocyte dysfunction associated with high levels of plasma TNF-α and early death in C57BL/6 mice. *J Immunol.* 163, 2089–2097.

Mason, S., Dubey, J. P., Smith, J. E., and Boag, B. (2015). *Toxoplasma gondii* coinfection with diseases and parasites in wild rabbits in Scotland. *Parasitology* 142, 1415–1421. doi: 10.1017/S003118201500075X

Meirelles, M. N., and Souza, W. de (1985). Killing of *Trypanosoma cruzi* and *Leishmania mexicana*, and survival of *Toxoplasma gondii*, in chicken macrophages *in vitro*. *J Submicrosc Cytol.* 17, 327–334.

Mordue, D. G., and Sibley, L. D. (1997). Intracellular fate of vacuoles containing *Toxoplasma gondii* is determined at the time of formation and depends on the mechanism of entry. *J Immunol.* 159, 4452–4459.

Morisaki, J. H., Heuser, J. E., and Sibley, L. D. (1995). Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. *J Cell Sci.* 108, 2457–2464.

Mota, M. M., and Rodriguez, A. (2001). Migration through host cells by apicomplexan parasites. *Microbes Infect.* 3, 1123–1128. doi: 10.1016/s1286-4579(01)01473-3

Qureshi, M. A., Heggen, C. L., and Hussain, I. (2000). Avian macrophage: effector functions in health and disease. *Dev Comp Immunol.* 24, 103–119. doi: 10.1016/s0145-305x(99)00067-1

Renter ía-Sol ís, Z., Zhang, R., Taha, S., and Daugschies, A. (2020). A modified method for purification of *Eimeria tenella* sporozoites. *Parasitol Res.* doi: 10.1007/s00436-020-06602-w

Rifaat, M. A., Salem, S. A., Azab, M. E., el-Razik, I. A., Safer, E. H., Beshir, SR, and el-Shennawy, S. F. (1984). Experimental concomitant toxoplasma and malaria infection in rats. *Folia Parasitol (Praha).* 31, 97–104.

Ryning, F. W., and Remington, J. S. (1978). Effect of cytochalasin D on Toxoplasma gondii cell entry. *Infect Immun.* 20, 739–743.

Sibley, L. D. (2010). How apicomplexan parasites move in and out of cells. *Curr Opin Biotechnol.* 21 (5), 592–598. doi: 10.1016/j.copbio.2010.05.009

Sibley, L. D., Weidner, E., and Krahenbuhl, J. L. (1985). Phagosome acidification blocked by intracellular *Toxoplasma gondii*. *Nature.* 315, 416-419. doi: 10.1038/315416a0

Soares Medeiros, L. C., Gomes, F., Maciel, L. R., Seabra, S. H., Docampo, R., Moreno, S., Plattner, H., Hentschel, J., Kawazoe, U., and Barrabin, H. (2011). Volutin granules of *Eimeria* parasites are acidic compartments and have physiological and structural characteristics similar to acidocalcisomes. *J Eukaryot Microbiol.* 58, 416–423. doi: 10.1111/j.1550-7408.2011.00565.x

Thabet, A., Alnassan, A. A., Daugschies, A., and Bangoura, B. (2015). Combination of cell culture and qPCR to assess the efficacy of different anticoccidials on Eimeria tenella sporozoites. *Parasitol Res.* 114, 2155–2163. doi: 10.1007/s00436-015-4404-4

Tierney, J., Gowing, H., van Sinderen, D., Flynn, S., Stanley, L., McHardy, N., Hallahan, S., and Mulcahy, G. (2004). *In vitro* inhibition of *Eimeria tenella* invasion by indigenous chicken *Lactobacillus* species. *Vet Parasitol.* 122, 171–182. doi: 10.1016/j.vetpar.2004.05.001

Trout, J. M., and Lillehoi, H. S. (1993). Evidence of a role for intestinal CD8+ lymphocytes and macrophages in transport of *Eimeria acervulina* sporozoites. *The J Parasitol.* 79, 790–792.

Vervelde, L., Vermeulen, A. N., and Jeurissen, S. H. M. (1996). *In situ* characterization of leucocyte subpopulations after infection with *Eimeria tenella* in chickens. *Parasite Immunol.* 18, 247–256. doi: 10.1046/j.1365-3024.1996.d01-94.x

Wiersma, H. I., Galuska, S. E., Tomley, F. M., Sibley, L. D., Liberator, P. A., and Donald, R. G. K. (2004). A role for coccidian cGMP-dependent protein kinase in motility and invasion. *Int J Parasitol.* 34, 369–380.

Zhang, R., Thabet, A., Hiob, L., Zheng, W., Daugschies, A., and Bangoura, B. (2018). Mutual interactions of the apicomplexan parasites *Toxoplasma gondii* and *Eimeria tenella* with cultured poultry macrophages. *Parasit Vectors.* 11: 453. doi: 10.1186/s13071-018-3040-0

2.2.5 Publication 5: A modified method for purification of *Eimeria tenella* sporozoites.

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I. Concept

Runhui Zhang and Zaida Renter á-Sol ś were responsible for the idea and design of this work

II. Investigation

Runhui Zhang prepared and collected samples and conducted experiment verification

III. Manuscript

Runhui Zhang improved manuscript and took part in the published work.

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PROTOZOOLOGY - SHORT COMMUNICATION

A modified method for purification of *Eimeria tenella* sporozoites

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Abstract
Coccidiosis is an economically important gastrointestinal disease in domestic fowl. *Eimeria* species are the causative agents of avian coccidiosis. Current challenges in management and prevention of eimeriosis enhance the need for research in this field. Sporozoite purification is a necessary step for *Eimeria* spp. in vitro infection models. Current alternatives such as DE-52 anion exchange chromatography and Percoll gradient require time and resources. We present a modified protocol consisting on vacuum filtration of sporozoites using a disposable 5-μL filter. Yield percentages were similar to those reported for Percoll gradient purification. By reducing time and efforts during sporozoite purification, it could be possible to increase resources in other areas of *Eimeria* studies.

Keywords *Eimeria* · Coccidiosis · Chicken · Sporozoite · Purification · Excystation

Introduction

Coccidiosis is one of the most important diseases of the poultry industry. It can cause economic losses of over 800 million US dollars annually worldwide (Shirley et al. 2007). *Eimeria* spp. are the cause of coccidiosis in domestic fowl. These parasites cause gastrointestinal problems characterised by diarrhoea, weight loss, reduced egg production and in some cases death. In chickens, seven *Eimeria* spp. are commonly found in the field. They differentiate themselves in oocyst morphology, virulence and area of infection in the digestive system. Additionally, infection with *Eimeria* spp. can promote opportunistic infections by other pathogens like bacteria (Collier et al. 2008). *Eimeria* oocysts are highly tolerant to the environment, which makes control measures difficult. Additionally, the common prophylactic use of anticoccidial feed additives has led to wide spread anticoccidial resistance (Stephan et al. 1997). *Eimeria tenella* is one of the most frequent and pathogenic *Eimeria* spp. in domestic fowl (Blake et al. 2015). It can cause haemorrhagic caecal disease with fatal outcome in some cases. Given its importance, *E. tenella* is commonly studied and used as a model for chicken coccidiosis (Blake et al. 2015, Hiob et al. 2017, Thabet et al. 2017, Zhang et al. 2018). Nevertheless, there is still a large need for more research within this field. A valuable tool regularly used as an alternative to in vivo model is in vitro infection assay (Dimier-Poisson et al. 2004, Thabet et al. 2017). Purification of *Eimeria* sporozoites is an important step before cell culture infection. There are currently some purification methods available (Schmatz et al. 1984, Dulski and Turner 1988, Zhang et al. 2015). Purification of *E. tenella* sporozoites by DE-52 anion exchange chromatography is a method regularly used during in vitro studies (Schmatz et al. 1984). Nevertheless, this technique involves time and additional materials. Similarly, the use of Percoll gradients also requires time for gradient preparation (Dulski and Turner 1988). In this study, we present a simple alternative for *E. tenella* sporozoite purification.

Zaida Rentería-Solís and Runhui Zhang contributed equally to this work.

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Material and methods

Eimeria tenella Houghton strain (kindly provided by Prof. Dr. D. P. Blake, Royal Veterinary College, University of London, UK) was passaged in healthy 10-day old chickens. Oocysts

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were purified from the faeces of the chickens following a modified protocol (Eckert et al. 1995). Briefly, faecal samples were collected and transferred to a 5-L plastic bucket. Thereafter, 2 volumes of tap water were added. Then, samples were homogenised with a hand blender (Braun, Frankfurt, Germany) until the mixture was homogeneous. The homogenate was filtered through a 250-μm-pore-size sieve and the filtrate transferred to a 2-L cylinder and sedimented overnight. Afterwards, the supernatant was discarded, and the sediment was resuspended in saturated saline solution. Followed by centrifugation of the resuspension at 1300×*g* for 10 min. Afterwards, suspended oocysts were collected and washed with tap water by centrifugation at 1300×*g* for 10 min. Finally, purified oocysts were collected and incubated for sporulation in 4% potassium dichromate solution at room temperature for 48 h. Sporulated oocysts were stored at 4 °C until further use.

The entire protocol was performed in triplicate with a total amount of 1.5×10^5 oocysts per replicate. Before excystation, sporulated oocysts were cleaned from the potassium dichromate through centrifugation. Briefly, oocysts were centrifuged at 1300×*g* for 10 min at room temperature (RT). Afterwards, the supernatant was discarded and the pellet was resuspended in sterile PBS (pH 7.0) and centrifuged again at 1300×g for 10 min at RT. This centrifugation step was repeated twice or until the supernatant was clear. Oocysts' surface sterilisation was performed as follows: after the last centrifugation of the previous step, the pellet was resuspended in 12% sodium hypochlorite (Carl-Roth, Karlsruhe, Germany) and incubated in a tube mixer at room temperature for 10 min. Immediately afterwards, the oocysts were centrifuged at 2500×*g* for 3 min. After centrifugation, the white cloudy layer formed at the top of the supernatant was transferred to clean 50-mL tube and resuspended in sterile PBS (pH 7.0). Oocysts were cleaned from sodium hypochlorite through vacuum filtration using a sterile1-μm Pluristrainer® filter (Pluriselect, Leipzig, Germany) mounted on a sterile connector ring (Pluriselect, Leipzig, Germany). After filtration, the filter was washed 3 times with sterile PBS (pH 7.0) in order to recover the oocysts. The filtration step was repeated once more. After the second filtration, recovery of oocysts was done using sterile PBS with pH 7.6–8.0. Alternatively, the oocysts can also be cleaned from the sodium hypochlorite by centrifugation at 1300*g* for 10 min, followed by 3 wash cycles with PBS at 1300*g* for 10 min. The recovered oocysts were immediately transferred to a 15-mL falcon tube with 0.5-mm sterilised glass beads (Carl Roth, Karlsruhe, Germany). The glass beads filled the tube up to the 0.5- to 1-mL mark. Once added to the tube with glass beads, the oocyst suspension was filled with sterile PBS (pH 7.6–8.0) up to 4 mL. Release of the oocysts' wall was performed by vortexing the tubes for 3 cycles of 20 s. After each 20 s cycle, the sporocyst-oocyst ratio was examined using a light microscope. If necessary, an additional 20-s cycle was performed in order to maximise the number of released sporocysts. Afterwards, the supernatant with released sporocysts was transferred to a 15-mL falcon tube. The glass beads were washed 2–3 times with 3 mL of sterile PBS (pH 7.6–8.0) to collect remaining sporocysts. Later on, sporocysts were centrifuged at 2500×*g* for 10 min at RT. The supernatant was discarded and the pelleted sporocysts were enzymatically excysted. Briefly, sporocysts were incubated with 0.25% trypsin (Biochrom AG, Berlin, Germany) and 4% sodium taurocholic acid (Sigma, Taufkirchen, Germany) in sterile PBS (pH 7.6–8.0) at 41 °C for 60 to 90 min. Monitoring of excystation rate was performed every 30 min with light microscopy. After incubation, free sporozoites were transferred to 50 mL of 1% glucose (Carl Roth GmbH, Karsruhe, Germany) in sterile PBS (pH 7.0) previously warmed to 41 °C. Immediately afterwards, sporozoites were purified by vacuum filtration, using a 5-μm Pluristrainer® filter (Pluriselect, Leipzig, Germany) mounted on a sterile connector ring (Pluriselect, Leipzig, Germany). To reduce the amount of sporocyst residue, sporozoites can also be filtered by gravity. For this option, the sterile connector ring is superfluous. After filtration, sporozoites are washed from the glucose solution by centrifugation at 3200×*g* for 10 min at RT. Right thereafter, the supernatant is carefully removed and the sporozoites are resuspended in the appropriate infection medium. Finally, sporozoites are counted under a light microscope using a Neubauer chamber (depth 0.100 mm, Paul Marienfeld GmbH, Lauda-Königshofen, Germany).

Sporozoite viability after purification was assessed according to Thabet et al. (2015). Briefly, Madin-Darby Bovine Kidney Cells (MDBK) were seeded in 24-well plates with Dulbecco's Modified Eagle's Medium (DMEM) with 5% foetal bovine serum (FBS), 100 IU penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B. Cells were incubated at 37 °C and 5% CO_2 until they reached 80 to 90% confluence. Cells were infected with freshly purified *E. tenella* sporozoites (5×10^4/well). The negative control group (NC) consisted of uninfected MDBK cells. All groups were performed in triplicate. Following infection, all groups of cell cultures were incubated at 41 °C and 5% CO_2 for 24 h. After 24 h, cells were washed 3 times with sterile PBS (pH 7.0) and fresh DMEM was added. After incubation at 41 °C for 96 h DNA was extracted from the cells using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Quantification of *E. tenella* genomic copies was performed in triplicate using a real-time PCR assay. Additionally a non-template control (NTC) consisting of nuclease-free water was added in triplicate to the assay. The primers ETF 5′-TGGAGGGGATTATG AGAGGA-3′ and ETR 5′-CAAGCAGCATGTAA CGGAGA-3′ were used to amplify a 147-bp fragment of the *E. tenella* internal transcribed spacer 1 (ITS-1) gene using a SYBR Green-based assay (Thermo Scientific, Darmstadt,

Germany) according to Kawahara et al. (2008). Finally, the number of gene copies was calculated from the qPCR data by using a standard curve prepared from a serial dilution of cloned ITS-1 gene fragment according to Thabet et al. (2015).

Results and discussion

Sporozoites recovered using the described protocol were clean and motile. After oocysts sterilisation with 12% sodium hypochlorite, yield efficacy was calculated for every further step of the procedure (Table 1). Overall, a yield of 35.69 ± 8.93% was recorded. Parasite loss was highest after grinding of oocysts with a yield mean of 55.55 ± 9.17% (Table 1). A low loss of sporozoites was calculated for the purification step with a mean value of 3.33%. Sporozoites collected by the current procedure were viable as assessed by successful in vitro cell invasion and replication. An initial number of $1.21 \pm 0.39 \times 10^5$ gene copies of the selected *E. tenella* gene fragment was determined by qPCR in 3 μL of total DNA after 96 h of incubation of MDBK following infection with 4×10^4 sporozoites.

We present a modified protocol for *E. tenella* sporozoite purification. An earlier version of this protocol has been successfully implemented in in vitro studies (Zhang et al. unpublished data, Rentería-Solís et al. unpublished data). Purification of *E. tenella* sporozoites can also successfully be performed by other protocols such as DE-52 anion exchange chromatography (Schmarz et al. 1984), Percoll gradients (Dulski an Turner 1988, Thabet et al. 2015) or with a 1400-mesh filter (Zhang et al. 2015). The protocol described in this report uses a plastic disposable 5-μm filter that perfectly fits to a 50-mL falcon tube. In this study, the Pluristrainer® model from Pluriselect (Leipzig, Germany) was used; however, similar products by other manufacturers exist in the market and are probably equally suited. By using a disposable 5-μL filter, there is no need of applying additional purification steps. This reduces the costs and time spent on sporozoite purification significantly thus saving resources in laboratory approaches.

Dulski and Turner (1988) reported a total yield of 39% of sporozoites using Percoll gradient for purification. Similar efficacy was obtained with the method presented in this study with 35.69 ± 8.93% of sporozoites recovered. Interestingly, Schmatz et al. (1984) reported a recovery of between 94 and 100% sporozoites after DE-52 anion exchange chromatography. However, the authors do not specify if the recovery percentage is calculated based on the initial amount of oocysts or in relation to the number of sporozoites collected during the previous step of purification. In our study, only 55 ± 9.17% of sporocysts were recovered after grinding of the oocysts' wall with glass beads. Similar results have been reported by Dulski and Turner (1988) with 51% of recovered sporocysts. Mechanical grinding with glass beads is not likely to destroy every single oocyst's wall. Excess of mechanical impact bears the risk of destruction of already liberated sporocysts and sporozoites. Alternatively, a mortar and pestle can also be used to grind oocysts instead of glass beads. Doran and Farr (1962) report a recovery of 30–65% of available *E. acervulina* sporocysts using the mortar method. Furthermore, in our new protocol, an average of only 3.33% of excysted sporozoites was lost during purification after sporocysts excystation. These results could be similar to those reported by Schmatz et al. (6 to 0% loss) if their recovery percentage was calculated from the total of excysted sporozoites. Dulski and Turner (1988) described only 1% loss of sporozoites after Percoll purification. However, that method comprises two Percoll gradient centrifugations of 20 min each which is time-consuming.

Research on chicken *Eimeria* spp. brings insights into coccidian metabolism, genetics, epidemiology and host-parasite interaction (Györke et al. 2013, Blake et al. 2015, Zhang et al. 2015). These developments could translate into improved therapies or preventive measurements. As ethically responsible study designs, in vitro models are pivotal to fulfil the goals of the 3R principle in animal research (Russell and Burch 1959). *Eimeria* sporozoites are commonly used in in vitro studies (Zhou et al. 2013, Thabet et al. 2015, Zhang et al. 2015, Bussière et al. 2018, Zhang et al. 2018). Sporozoites are the first cell invasive stage during eimeriosis. Therefore, any research on in vitro features of these coccidia depends on availability of viable sporozoites.

Table 1 Parasite recovery and yield efficacy

Step	Recovered parasitic stage	Quantity of parasites after each step[a]	Yield (%)[a,b]
Cleaning of oocysts with 1-μm filter[c]	Oocysts	$1.19 \pm 0.13 \times 10^5$	79.44 ± 9.17
Oocysts mechanical grind with glass beads	Sporocysts	$3.33 \pm 0.55 \times 10^5$	55.55 ± 9.17
Excystation	Sporozoites	$4.46 \pm 0.9 \times 10^5$	39.02 ± 7.50
Sporozoites purification with 5-μm filter	Sporozoites	$4.28 \pm 1.07 \times 10^5$	35.69 ± 8.93

[a] Quantities and yields reported are the means ± standard deviations of triplicate

[b] Yield percentages calculated from an initial amount of 1.5×10^5 oocysts

[c] Yield percentages of earlier steps during oocysts cleaning were not calculated

The development of a time saving and economic alternative to current methods for sporozoite purification could increase the interest in eimeriosis research. Furthermore, this protocol was established in *E. tenella*. Therefore, applications of this modified method in further *Eimeria* species should be encouraged and likewise reported.

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Compliance with ethical standards

Ethical approval All procedures performed involving animals were in accordance with the ethical standards of the local authorities (Landesdirektion Sachsen, permit no. A04/19).

Conflict of interest The authors declare that they have no conflict of interest.

References

Blake DP, Clark EL, Macdonald SE, Thenmozhi V, Kundu K et al (2015) Population, genetic, and antigenic diversity of the apicomplexan *Eimeria tenella* and their relevance to vaccine development. PNAS:E5343–E5350

Bussière FI, Niepceron A, Sausset A, Esnault E, Silvestre A, Walker RA, Smith NC, Quére P, Laurent F (2018) Establishment of an *in vitro* chicken epithelial cell line model to investigate *Eimeria tenella* gamete development. Parasit Vectors 11:44. https://doi.org/10.1186/s13071-018-2622-1

Collier CT, Hofacre CL, Payne AM, Anderson DB, Kaiser P, Mackie RI, Gaskins HR (2008) Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Vet Immunol Immunopathol 122:104–115

Dimier-Poisson IH, Bout DT, Quére P (2004) Chicken primary enterocytes: inhibition of *Eimeria tenella* replication after activation with crude interferon-gama supernatants. Avian Dis 48:617–627

Doran DJ, Farr MM (1962) Excystation of the poultry coccidium, *Eimeria acervulina*. J Protozool 9:154–161

Dulski P, Turner M (1988) The purification of sporocysts and sporozoites from *Eimeria tenella* oocysts using Percoll density gradients. Avian Dis 32:235–239

Eckert J, Braun R, Shirley MW, Coudert P (1995) Biotechnology guidelines on techniques in coccidiosis research. COST European Cooperation in the field of Scientific and Technical Research, Brussels, Belgium

Györke A, Pop L, Cozma V (2013) Prevalence and distribution of *Eimeria* species in broiler chicken farms of different capacities. Parasite 20:50

Kawahara F, Taira K, Nagai S, Onaga H, Onuma M, Nunoya T (2008) Detection of five avian *Eimeria* species by species-specific real-time polymerase chain reaction assay. Avian Dis 52:652–656

Russell WMS, Burch RL (1959) The principles of humane experimental technique. Methuen, London

Shirley MW, Smith AL, Blake DP (2007) Challenges in the successful control of avian coccidia. Vaccine 25:5540–5547

Schmatz DM, Crane MST, Murray PK (1984) Purification of *Eimeria* sporozoites by DE-52 anion exchange chromatography. J Protozool 31:181–183

Thabet A, Alnassan AA, Daugschies A, Bangoura B (2015) Combination of cell culture and qPCR to assess the efficacy of different anticoccidials on *Eimeria tenella* sporozoites. Parasitol Res 114: 2155–2163

Thabet A, Zhang R, Alnassan AA, Daugschies A, Bangoura B (2017) Anticoccidial efficacy testing: *in vitro Eimeria tenella* assays as replacement for animal experiments. Vet Parasitol 233:86–96. https://doi.org/10.1016/j.vetpar.2016.12.005

Zhang R, Thabet A, Hiob L, Zheng W, Daugschies A, Bangoura B (2018) Mutual interactions of the apicomplexan parasites *Toxoplasma gondii* and *Eimeria tenella* with cultured poultry macrophages. Parasit Vectors 11:453. https://doi.org/10.1186/s13071-018-3040-0

Zhang Y, Zheng MX, Xu ZY, Xu HC, Cui XZ, Yang SS, Zhao WL, Li S, Lv QH, Bai R (2015) Relationship between *Eimeria tenella* development and host cell apoptosis in chickens. Poult Sci 94:2970–2979. https://doi.org/10.3382/ps/pev293

Zhou Z, Wang Z, Cao L, Hu S, Zhang Z, Qin B, Guo Z, Nie K (2013) Upregulation of chicken TLR4, TLR15 and MyD88 in heterophils and monocyte-derived macrophages stimulated with *Eimeria tenella in vitro*. Exp Parasitol 133:427–433. https://doi.org/10.1016/j.exppara.2013.01.013

3 Overreaching discussion

Probably due to the mostly sub-clinical course of toxoplasmosis in chicken only few studies focused on the multiple *in vivo* and *ex vivo* alterations that may occur during co-infection with *Eimeria* species and *T. gondii* in poultry, so far. HIOB et al. (2017) reported findings obtained in experimentally co-infected chicken and showed interaction between both pathogens regarding host immune response as well as development of parasite burden during the co-infection. In the current work these studies were extended to investigation of potential interactions *in vitro* with a particular focus on the avian innate immune system using primary macrophages as host cells.

3.1 Chicken primary monocyte-derived macrophages are suitable to study *in vitro* co-infection by both *T. gondii* tachyzoites and *E. tenella* sporozoites (Publication 1, 2, 5 and Manuscript 3-4).

Mammalian macrophages have been used to study the growth of *T. gondii* and related immune mechanism for a long time (VISCHER and SUTER 1954). *T. gondii* is capable to propagate in mammalian and avian macrophages. Although MALKWITZ et al. (2013) reported that *T. gondii* replication was more efficient at 37 ℃ than at 40 ℃ in chicken primary macrophages, they suggested that normal avian body temperature (approximately 40 ℃) should be considered in the avian model with respect to the physiological host reactivity and metabolism. In this study, a major proportion of *T. gondii* tachyzoites were eliminated in chicken primary macrophage cultures before 12 hpi, but considerable replication was observed thereafter, i.e. within 12 to 24 hpi (Publication 1, 2 and Manuscript 3). In contrast, *E. tenella* appear less adapted to replication in chicken primary macrophages which is in accordance to the study of LONG and ROSE (1976), who reported poor growth of *E. tenella* in peritoneal macrophages. However, *E. tenella* reproduction was observed in our model until 72 hpi. A higher increase in parasites numbers was seen in the cultures exposed to a higher infection dose in the present study. Although macrophages thus displayed a (limited) ability to support *E. tenella* development and are rather known to serve as “trojan horses”, the present study confirmed that they are not adequate natural host cells for *E. tenella* reproduction.

Previous experiments were often performed in epithelial cells which are considered to be a suitable

host cell type for *in vitro* studies on *E. tenella*. In fact, MDBK cells have been applied frequently and successfully to study both *T. gondii* and *E. tenella* multiplication in mono-infection models (DIAB and EL-BAHY 2008). In the present study (Publication 2), MDBK cells were also used to investigate the parasite growth during co-infection for comparison to primary chicken macrophages. The optimal temperature for parasite cultivation is 37 ℃ for *T. gondii* but 41 ℃ for *E. tenella* in the MDBK cell line (HUGHES and FLECK 1986). In this study, multiplication of *T. gondii* tachyzoites was distinctly lower in MDBK cells than in chicken macrophages at 41 ℃. Conversely, the growth of *E. tenella* appeared significantly higher than in chicken macrophages at 24 hpi. Considering potential alteration of host cell metabolism/reactivity at 37 ℃ in the avian model, incubation temperature representing normal chicken body temperature was suitable for both parasites growth in the primary cell model evaluated in the current study.

In the current study *T. gondii* RH tachyzoites were used due to their capacity of *in vitro* reproduction and most active intracellular multiplication (YANG et al. 1998). *E. tenella* Houghton sporozoites were selected because the Houghton strain is a well-established laboratory tool applied by many research groups worldwide (CHAPMAN and SHIRLEY 2003). Use of the sporozoite stage for *E. tenella* has three advantages: a. sporozoites are the initial invasive stages in the *Eimeria* life cycle, thus decisive for the course of the infection; b. they can be easily acquired from oocysts (ECKERT et al. 1992); c. they can be easily differentiated by their morphology from *T. gondii* tachyzoites. Genetically modified parasites expressing fluorescence are broadly applied to track parasite activities or for drug screening (GUBBELS et al. 2004). *T. gondii* expressing green fluorescent protein (GFP) and *E. tenella* expressing yellow fluorescent protein (YFP) were used to evaluate parasite development. Additionally, an improved protocol was developed in this study in order to remove contamination and to produce highly purified and germ-free *E. tenella* sporozoites (Publication 5).

In a recent study (MALKWITZ et al. 2013), *T. gondii* ME49 strain infection destroyed almost all primary macrophages and numerous free tachyzoites were found at 72 hpi when infected with MOI (“multiplicity of infection”) of 7.5 tachyzoites per host cell. A macrophage-parasite ratio of 3:1 was proposed for studying infection by *T. gondii* RH strain in mammal macrophages (ROBBEN et al. 2004). Because of the faster growth rate of *T. gondii* RH strain as compared to ME49 strain (SAEIJ

et al. 2005), an approximate MOI of 2:1 to 4:1 (i.e., 2.5 x 10^5 to 5 x 10^5 tachyzoites / well) was selected in our experiments with the *T. gondii* RH strain. In order to establish comparable infection conditions, the same MOI of 2:1 and 4:1 was selected for *E. tenella* sporozoite infections. Furthermore, simultaneous and sequential infections were both carried out to investigate the interaction between these two parasites and their host cells. *In vitro* infection experiments were conducted in 24-well plates for simultaneous and sequential infection (Publication 2 and Manuscript 3). For time lapsing imaging, cell cultures at the same MOI in a microincubation system was chosen (Manuscript 4). Investigation of host-parasite interaction was archived with these experimental settings in the whole period of own co-infection model, e.g. the replication as well as reproduction of both parasites and appropriate numbers of host cells.

3.2 Parasite development of both *T. gondii* and *E. tenella* is mutually modulated in co-infected cell cultures (Publication 2 and Manuscript 3-4).

HUGHES and FLECK (1986) reported that *T. gondii* displayed different growth dynamics depending on the type of cell cultures used. SUNDERMANN and ESTRIDGE (1999) found that less than 10 % of *T. gondii* RH tachyzoites could penetrate human foreskin fibroblast (HFF) cells at 3 hpi. Similarly, at the early stage of my own infection experiments, approximately 10 % of *T. gondii* tachyzoites were located intracellularly in all *T. gondii*-infected cultures at 2 hpi (Publication 2 and Publication 3). Interestingly, *T. gondii* showed a different rate of penetration at different initial infection doses and the lowest number of intracellular parasites was seen in the co-infected group CI at 2 hpi. In the study of sequential co-infection, significantly low numbers of *T. gondii* were found in groups CI and LPS compared to the *T. gondii* mono-infections at 2 hpi (Manuscript 4). Assumingly, most tachyzoites did not successfully penetrate into the MMs but were rather incorporated by phagocytosis. This is in accordance to the study of MORISAKI, et al. (1995), most of effete parasites were internalized in murine macrophages by phagocytosis.

Cytokine mRNA expression such as IL-1β and iNOS were highly enhanced 2 hours after applying *E. tenella* merozoites to chicken HTC macrophages (CHOW et al. 2011). This indicates that host signaling and immune reaction of macrophages might be altered by *E. tenella* infection, which in our co-infections could lead to phagocytic fusion or extracellular death of *T. gondii* instead of active

macrophage penetration. This study showed that *E. tenella* infection impaired the active penetration of *T. gondii* into chicken macrophages in both simultaneous and sequential infection.

In general, *T. gondii* continues to divide every 8-10 h in the host cell eventually lysing the host cell within 24-48 hpi (MORDUE and SIBLEY 1997). Accordingly, *T. gondii* showed earliest replication in co-infection as well as mono-infection as analysed by CLSM at 12 hpi. However, compared to the initial infection dose only 50 % of *T. gondii* DNA copy numbers were measured in chicken macrophage cultures in mono-infected cultures at 12 hpi with a distinct copy number increase and thus replication afterwards (Manuscript 3).

In the co-infected macrophage cultures, *T. gondii* DNA copy numbers remained low irrespective infection rate and declined at 12 hpi to lowest, however not significantly reduced values as compared to mono-infection. The quantities of *T. gondii* showed a marked increase in all *T. gondii*-infected groups except group LPS from 24 hpi onwards. Interestingly, it appears important that the tachyzoites invade MMs before *E. tenella* infection for success survival of *T. gondii*. *T. gondii* in group CI showed a great ability of replication during simultaneous infection whereas no significant difference was observed during sequential infection while compared between mono- and co-infection. However, MOI seems to play a role in parasite survival in cell cultures. Live-cell imaging of 100 single host cells co-infected by *T. gondii* (MOI of 1-2 tachyzoites per cell) and *E. tenella* demonstrated that *T. gondii* in co-infected cultures showed a similar survival rate as in LPS-activated macrophage cultures. However, qPCR results of cell cultures showed significantly higher DNA copies following co-infection than in group LPS. Therefore, the numbers of macrophages available for parasite replication may also play a role in replication of *T. gondii*.

At 48 and 72 hpi, considerable multiplication of *T. gondii* was seen in cell culture irrespective of co- or mono- infection. MEIRELLES and DE (1985) likewise showed that *T. gondii* was able to well replicate in chicken blood monocyte-derived macrophages. Both *E. tenella* and LPS affected *T. gondii* invasion. This may indicate that there is an activation of innate immune reaction by *E. tenella* although this has only limited effect on *T. gondii* when it is well protected by PV during intracellular development.

Compared to *T. gondii* tachyzoites, *E. tenella* sporozoites appear not to survive and multiple well in primary chicken macrophages at the early stage of infection. Similar to previous *in vitro Eimeria* studies (LONG and ROSE 1976; TAUBERT et al. 2009), most of sporozoites entered chicken primary macrophages possibly by phagocytosis within 2 hpi in this study (Manuscipt 3). Only less than approximately 20 % of initially applied *E. tenella* DNA copies were detectable at 2 hpi in spite of generally higher initial infection efficiency than *T. gondii* described above. CORNELISSEN et al. (2009) reported that mixed *in vivo Eimeria* species infection resulted in similar intestinal damages and replication compared to mono-species infection in chickens. The study of HIOB et al. (2017) indicated that *Eimeria* spp. merogony was increased in chickens co-infected with *T. gondii* whereas no significant difference of oocyst excretion was found compared to mono-infection. Interestingly, own experiments showed that *T. gondii* caused low inhibition of macrophage phagocytosis in group CI. This may reflect significantly higher infectivity of *E. tenella* at 6 hpi during simultaneous infection with *T. gondii* than during mono-infection. Moreover, both simultaneous and sequential co-infection displayed slightly higher infectivity of both parasites in co-infection cultures compared to mono-infection with the same infection dose at 6 hpi. While *T. gondii* replication was particularly obvious in the co-infected cell culture after 24 hpi, only a small number of intra-macrophage *E. tenella* sporozoites developed to the intracellular meront stages not earlier than12 hpi (Publication 2). In general, *E. tenella* replication was more efficient in simultaneously co-infected cultures as compared to mono-infected cultures. Consequently, it is possible that *T. gondii* supports or extends at least partially *E. tenella* survival in co-infected cells.

3.3 Phagocytosis capacity and regulation of cytokines by chicken primary macrophages are mutually modulated by *T. gondii* and *E. tenella* (Publication 2 and Manuscript 3).

Macrophages are the most important immune cell populations and act as the first line of the chicken innate immune response (QURESHI 2003). Therefore, efficiency of macrophage phagocytosis and relevant cytokines during coccidial infections were evaluated here under experimental *in vitro* conditions.

Phagocytosis plays a crucial role in engulfment and initialization of pathogens by chicken

macrophages during early infection (QURESHI et al. 2000). After phagocytosis induction by pH-sensitive Zymosan bioparticles, isolated chicken primary macrophage cultures revealed a high capacity of phagocytosis. Our experiments demonstrated early (at 2 hpi) phagocytosis activation in on average 72 % of primary macrophages following bioparticles stimulation. Reactivity of macrophages decreased over time to approximately 36 % at 24 hpi. KAPELLOS et al. (2016) stated that cell culture density and activation duration were two major factors affecting the uptake of fluorescent particles by murine bone marrow-derived macrophages. GARRIDO et al. (2018) showed recently that 25% of HD 11 cells were activated by bioparticles following a 16 h dimethylsulfoxid treatment. The current study connfirmed the statement of KAPELLOS et al. (2016), that a decline of activation of phagocytosis was seen after LPS polarization (Publication 3). Therefore, our chicken primary macrophage model with the observed dynamics of macrophage activation appears to be a suitable model to investigate both mono- and co-infection of *T. gondii* and *E. tenella* at the early stage *in vitro*. However, the current findings (Publication 3) indicate that long-term phagocytosis studies require further optimization of the cultivation conditions for primary chicken macrophages.

For both, *T. gondii* and *E. tenella*, phagocytosis has been identified as a major route of entry into macrophages previously (GOREN 1977; ROSE and LEE 1977b). During the internalization, some parasites are able to actively invade the host cell and to avoid the fusion with phagosomes/endosomes/lysosomes by hiding within the PV (MORISAKI et al. 1995; BUTCHER and DENKERS 2002). In the experiments observing the phagocytic capacity of macrophages for bioparticles (Publication 3), it was apparent that bioparticle phagocytosis was inhibited in all infected groups at 2 hpi. Interestingly, higher initial parasite infection doses (higher MOI) induced a more pronounced bioparticle phagocytosis inhibition during either *T. gondii* or *E. tenella* mono-infection. In contrast, inhibition showed significantly lower values in group CI in spite of application of an identical sum of *T. gondii* and *E. tenella* numbers compared to *T. gondii* mono-infection (group TH, MOI of 4 : 1) and *E. tenella* mono-infection (group EH, MOI of 4:1).

Effective phagocytosis and low growth of *E. tenella* sporozoites in primary macrophages further explains that macrophage-infiltrated lamina propria was frequently found in the intestine of *Eimeria*-infected chicken (VERVELDE et al. 1996). This may be linked to the low infectivity of *T.*

gondii in co-infected cultures as discussed above. At later observation time points, macrophage activation by bioparticles revealed marked differences between *T. gondii* and *E. tenella* mono-infected groups. Phagocytosis inhibition was particularly pronounced in *E. tenella* infected cultures. In contrast, macrophage bioparticle phagocytosis was less inhibited in group CI than in *E. tenella* mono-infected groups, which may be attributed to increased numbers of *T. gondii* at 6 hpi in group CI. *E. tenella* engulfment was increased in group CI compared to mono-infected cultures at the same time point due to more active macrophages, i.e. higher phagocytic activity for bioparticles.

At 12 hpi, though parasite replication seemed to be similar in the mono- and co-infection groups, co-infected cell cultures showed distinctly lower reaction to bioparticles than mono-infected cultures. In fact, co-infected cells did never simultaneously incorporate bioparticles as observed by CLSM.

Similar to the LPS polarization which was described earlier, co-infection likely triggered immune pathways such as cytokine expression (Publication 2) *in vitro* and stronger so than mono-infections did. Six types of cytokines produced by chicken macrophages were investigated in this study according to previous studies on coccidia infections (LIEBERMAN and HUNTER 2009; MILANOVA et al. 2016): Th2-supporting IL-6, Th1-supporting IL-12, IFN-γ-inhibiting IL-10, and innate immune response-related iNOS, TNF-α and IFN-γ (Publication 2).

Macrophages are essential to control protozoan infection by triggering the Th1 response (DENKERS et al. 2003). It was expected that Th2-supporting IL-6 is not significantly affected under the conditions of the current study since this cytokine is not related to major immunological reaction pathways to apicomplexan infections. In contrast, IFN-γ is a Th1-related cytokine that is considered to be an important factor in the replication inhibition of *T. gondii* and *E. tenella* (SUZUKI et al. 1988; LILLEHOJ and CHOI 1998). Previous *in vivo* studies (YUN et al. 2000; MILANOVA et al. 2016 and HIOB et al. 2017) demonstrated that IFN-γ mRNA levels were significantly up-regulated in the caecum of *E. tenella*-infected animals. HIOB et al. (2017) also showed that the level of IFN-γ increased during co-infection with these two investigated parasites at the early stage of infection. Although macrophages are not the main immune cells producing IFN-γ, macrophage-produced IFN-γ was reported to be of biological significance in the early phase of host response to pathogens (GESSANI and BELARDELLI 1998).

In the present study, the expression of IFN-γ was upregulated in response to *E. tenella* infection, whereas co-infection appeared to suppress respective mRNA expression. One IFN-γ function is to trigger macrophages to produce iNOS resulting in high levels of cytotoxic NO (DENKERS et al. 2003). LILLEHOJ and LI (2004) and ALLEN (1997) reported that significantly elevated levels of NO were induced in *E. tenella*-infected chicken. In contrast, GUILLERMO and DAMATTA (2004b) demonstrated that *T. gondii* inhibits NO production thus supporting survival of chicken macrophages. This is in line with our own observations that iNOS was distinctly upregulated in *E. tenella* mono-infection as compared to *T. gondii* mono-infection at 24 hpi. In this study, iNOS seemed to play a crucial role in controlling parasite replication and was particularly upregulated during co-infection.

IL-12 is essential during both the acute and chronic phase of *T. gondii* infection and linked to a reduction in parasite load (LETSCHER-BRU et al. 1998; BUTCHER and DENKERS 2002). However, infection by the *T. gondii* RH strain failed to trigger the production of IL-12 by murine macrophages at 24 hpi (ROBBEN et al. 2004), which is in general consistent with the results of the present study. However, upregulation of IL-12 was seen in primary macrophages infected with the higher dose of *T. gondii* (group TH) at 48 and 72 hpi.

Prior studies in the HTC cell line infected with *E. tenella* merozoites showed downregulation of IL-12 from 6 hpi to 24 hpi (CHOW et al. 2011). Conversely, own results indicate a distinct upregulation of IL-12 after low-dose *E. tenella* infection (group EL) at 24 hpi, and in all *E. tenella*-infected macrophage cultures after longer observation periods of 48 hpi or 72 hpi. Nonetheless, a marked enhancement in IL-12 expression was not observed in co-infected cultures, which is in contrast to recent *in vivo* studies (BANGOURA et al. 2014; HIOB et al. 2017). Therefore, we assume that macrophages are probably not the major source of IL-12 expression during co-infection.

IL-10 inhibits the production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α by monocyte-derived macrophages (SASAI et al. 2018). In toxoplasmosis, IL-10 is able to inhibit the capacity of IFN-γ to activate macrophages to eliminate *T. gondii* (GAZZINELLI et al. 1992), whereas WILLE et al. (2001) suggested that IL-10 did not contribute to the course of infection with

the *T. gondii* RH strain. In chicken coccidiosis, high expression of IL-10 was found *in vivo* as well as *in vitro* in activated monocyte-derived macrophages during *E. maxima* infection (HONG et al. 2006; ROTHWELL et al. 2004). In the only published *in vivo* co-infection study on *T. gondii* and *Eimeria* spp. in chickens, HIOB et al. (2017) showed that no significant level of IL-10 was elicited. In the present study, IL-10 mRNA upregulation was observed mainly during *T. gondii* infection with a delayed peak in the co-infected group CI that coincides with increased *E. tenella* replication. Therefore, it is supposed that, with the participation of multiple immune cells *in vivo*, chicken macrophages play a major role in regulation of IL-10 expression in the complex regulation of the innate immune response to *T. gondii* and / or *E. tenella*.

Expression of TNF-α was upregulated in all infected groups over the whole experimental period. Increased TNF-α has been described before in *E. tenella*-infected chicken macrophages (ZHANG et al. 1995a). Furthermore, BYRNES et al. (1993) measured the production of TNF-α by macrophages after infection with *Eimeria* and found a biphasic pattern, with a first peak of TNF-α expression related to disease pathogenesis and a second peak related to development of protective immunity. However, as recorded for IL-12, *T. gondii* is also able to block the TNF-α expression by LPS-activated macrophages (BUTCHER and DENKERS 2002). TNF-α was supposed to synergize with the anti-*T. gondii* effect provided by IFN-γ in mammalian macrophages (CHANG et al. 1990). In chicken monocyte-derived macrophages, a distinct increase of TNF-α mRNA expression was observed in all *T. gondii*-infected groups at 72 hpi with a peak during co-infection at the end of the study. This may indicate functional differences in mammalian and avian macrophages in their reaction to apicomplexan parasites. Therefore, we conclude that chicken macrophage TNF-α production is significantly triggered by both *E. tenella* and *T. gondii* infection though this reaction may not be sufficient to counteract parasite replication in our model.

3.4 Invasion/phagocytosis patterns of *T. gondii* in macrophage cultures are affected by co-infection with *E. tenella* in the early phase of parasite invasion (Manuscript 3-4).

Host cell invasion relies on parasite motility, host cell attachment and active penetration. Macrophages are unique targets for parasites since they are capable of protozoan recognition and phagocytosis upon host infection (QURESHI 2003). MORISAKI et al. (1995) reported that *T. gondii*

tachyzoites only took 15-20 s to finish the cell entry by active penetration. This process is even faster than macrophage phagocytosis. As described above, *T. gondii* showed low replications in both *E. tenella* and LPS activated macrophages at 6 hpi when compared to infection of non-activated macrophage cultures. Parasite motility and intracellular survival of *T. gondii* were evaluated to better understand this phenomenon by time lapsing imaging (Publication 4).

By time lapsing imaging, tracked *T. gondii* tachyzoites showed lowest motility in group LPS whereas significantly higher motility was seen in group CI until 2 hpi. Despite similar DNA copy numbers of *T. gondii* were detected in the respective cultures (CI, LPS) at 2 hpi, it appears that vital tachyzoites may still engage in completing host cell invasion in group CI. Parasite tracking analysis revealed that prolonged attachment of highly motile tachyzoites to macrophages particularly occurred in group CI.

In prior investigations most *T. gondii* tachyzoites remained adherent to murine macrophages that were treated with phagocytosis inhibitor (RYNING and REMINGTON 1978). As stated above, *E. tenella* infection was efficient in inhibiting phagocytosis by chicken macrophages. On the other hand, phagocytic inhibition was associated with a significant burden of both intracellular *T. gondii* and *E. tenella* at 2 hpi in the current study (Manuscript 3). Single *T. gondii* infection of host cells also reduced phagocytosis, but inhibition was slightly lower than if induced by *E. tenella* infection. Therefore, the longer adherence of T. *gondii* may have been caused by phagocytosis inhibition. Interestingly, both *E. tenella* sporozoites and passively engulfed *T. gondii* tachyzoites seem to cause phagocytosis inhibition. Additionally, cytokines are probably involved in coordinating macrophage phagocytosis (QURESHI 2003). However, because of limited data, further studies on cytokine regulation are essential to understand this aspect of the host innate immune response at the early stage of *E. tenella* and *T. gondii* co-infection.

In general, avian toxoplasmosis is a mild and mostly subclinical disease, although two clinical cases were reported resulting from secondary infection following primary viral infection in chicken (HEPDING 1939; GOODWIN et al. 1994). Transgenic *T. gondii* were suggested as a live vaccine vector against eimeriosis in chicken recently (ZOU et al. 2011). On the other hand, transgenic *E. tenella* were also considered as possible *Toxoplasma* vaccine vehicle in chicken (TANG et al. 2016). Specific Th1 mediated immune responses were elicited for both applications. For development of

such vaccines, it is important to better understand the effects on the host immune system for both parasites, either exposure as a single pathogen or in combination. The current study provides evidence that the response of macrophages to infection and parasite reproduction is modified during co-infection in the model of primary chicken monocyte-derived macrophage cultures. The reported data and procedures established in the current study may help to understand macrophage-mediated immune responses in co-infections of two related parasitic protozoa, *T. gondii* and *E. tenella*, that are supposedly rather frequent under natural exposure conditions.

4 Conclusions

In summary, an *in vitro* model of chicken monocyte derived macrophages infected with two apicomplexan parasites, monoxenous *E. tenella* and heteroxenous *T. gondii*, was established and evaluated over a study period up to 72 hpi. This study supports the hypothesis that simultaneous or successive exposure to both parasites leads to single species or dual infection of primary avian monocyte-derived macrophages, respectively. Monocyte-derived macrophages were confirmed as a suitable host cell type for *T. gondii* development. *E. tenella* sporozoites were also able to reproduce in this infection model even though with lower replication potential. Compared to the MDBK cell line, this model enabled better adjustment with acceptable growth of both parasites cultured under the same circumstances.

The results provided evidence of mutual host-parasite interaction affecting morphology and cytokine expression different between mono- and co-infected macrophages. Increased vacuolization and phagocytosis inhibition in co-infected macrophages demonstrates that these cells are seemingly more stressed due to exposure to dual parasite infection. Enhanced production of IL-10 and TNF-α by macrophages as observed in the current study is tightly linked to the interaction with other cells in the infection organisms. This has to be considered before functional conclusions are drawn.

Parasite-parasite interaction manifested early as postponed host cell invasion by *T. gondii* tachyzoites in macrophages exposed to *E. tenella* co-infection. *T. gondii* took part in enhancing the macrophages' phagocytosis capacity resulting in more intracellular *E. tenella* found during the early period of co-infection.

Under field conditions, it can be assumed that infections with both parasites occur frequently and probably in most cases sequentially. The observed differences in parasite reproduction between sequential and simultaneous infection underlines the influence of infection timing for both pathogens in a co-infection scenario. In field situations, *Eimeria* may thus potentially predispose chickens to a higher *T. gondii* burden if *T. gondii* infection is acquired subsequently. However, mutual influences of such infections depend on close timely proximity whereas it appears unlikely that long periods between exposure to the one and the other parasite will result in distinct interaction. However, the

model used in the current study does not allow conclusions on long term effects, and thus respective assumptions are in fact speculative at present.

However, chicken monocyte-derived macrophage cultures may at least partially reflect immune mechanism and parasite-parasite interactions that are active *in vivo* during co-infection as many findings reported here were in line with *in vivo* results obtained by HIOB et al. (2017). In general, the established cell culture system is suitable and can be utilized to study the reaction of macrophages to certain protozoan infections and, as shown in this work, also to evaluate dual protozoan infections in chickens.

Avian and mammalian immune systems are partially different in their response mechanisms towards protozoa. Therefore, this *in vitro* model may also provide a basis to investigate the interplay of other protozoa with chicken macrophages such as *Leishmania* (OTRANTO et al. 2010) and *Plasmodium* (YURAYART et al. 2017), which have been so far intensively studied in similar models in mammals only.

The current avian model provides the possibility to assess primary macrophage reactions in the presence of different pathogens. Respective knowledge will strengthen the awareness that co-infections play an important role under conditions of natural exposure and that alterations in host-parasite systems may influence disease outcome. However, host immune responses form a complex system with a multitude of interfering factors. Therefore, the current study represents an initial step to better understand host-parasite-parasite interactions in birds but is not intended to deliver final answers. Quite the contrary, the obtained data lead to as yet unanswered questions requiring further investigation, both under controlled *in vitro* conditions as well as in experimental *in vivo* studies or even in natural infections.

5 Summary

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***In vitro* co-infection studies on *Toxoplasma gondii* and *Eimeria tenella* in primary poultry macrophages**

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Keywords: *Toxoplasma gondii*, *Eimeria tenella*, co-infection, macrophages, *in vitro*, host-parasite interaction, parasite growth, host immune response, cell imaging.

Introduction: *Toxoplasma gondii* and *Eimeria tenella* are intracellular apicomplexan parasites that are widely distributed in chicken. Whereas infections with the zoonotic pathogen *T. gondii* are generally subclinical in poultry, *E. tenella* may cause severe disease and economical loss in chicken herds. Due to the high seroprevelences with both parasites in chicken, co-infections are likely to exist. Chicken macrophages are essential in the host innate immune response against these two protozoa. However, little is known about the host-parasite and parasite-parasite interaction in chicken macrophages during co-infection.

Objectives: A suitable *in vitro* model for both *T. gondii* and *E. tenella* infection in chicken primary macrophages was established and applied to study the course of infection in mono- or co-infection.

Animals, materials and methods: Monocyte-derived macrophages were isolated and purified from chicken blood. Co-infection with two *T. gondii* RH strain tachyzoites and two *E. tenella* Houghton strain sporozoites per cell were performed simultaneously or sequentially *in vitro*. Morphologic alterations of macrophages and parasite development were visualized by confocal laser scanning microscopy (CLSM) at 2, 6, 12, 24, 48 and 72 hours post infection (hpi). Parasite growth was evaluated by assessing expression of the 529-bp fragment of *T. gondii* and ITS-1 gene fragment of *E. tenella* by qPCR in parallel. Macrophage phagocytosis was stimulated by exposure to pH sensitive fluorescent bioparticles and quantified by a three-dimensional model using CLSM and Imaris® software at 2, 6, 12 and 24 hpi. Furthermore, infection-related cytokines (IL-6, IL-10, IL-12, iNOS,

TNF-α and IFN-γ) were evaluated by gene expression analysis at 24, 48, 72 hpi. The course of sequential infection was evaluated to determine cell invasion and survival of *T. gondii* in macrophages previously exposed to *E. tenella* sporozoites. Motility of invasive stages was tracked at the early phase of infection (within 20 hpi) by real time life cell imaging.

Results： By cell imaging, macrophages displayed distinctly immunologic activation and phagocytosis at 2 hpi and thereafter. Significant changes of morphology with increased cell vacuolation and detachment were observed on 24 hpi during co-infection. *T. gondii* tachyzoites adhered for more than 4 hours to host cells displaying high motility instead of cell entry during the early sequential co-infection. However, *T. gondii* showed better replication than *E. tenella* in co-infected macrophages from 24 hpi onwards. Co-infection caused inhibition of phagocytosis by macrophages and bioparticles were not incorporated into co-infected cells at 12 hpi by CLSM. By qPCR, it was shown that approximately 4-fold less *T. gondii* survived in sequentially co-infected cultures at 2 hpi as compared to mono-infection ($P < 0.05$). Replication of both parasites increased during simultaneous co-infection whereas only half numbers of replicated *T. gondii* was found in sequential co-infection compared to mono-infection at 24 hpi. At the end of the study period (72 hpi), *E. tenella* multiplication tended to double increase while *T. gondii* replication was not distinctly altered during co-infection compared to 48 hpi. Expression of mRNA for iNOS at 48 hpi, for TNF-α at 72 hpi and for IL-10 at 48 and 72 hpi was significantly elevated during co-infection compared to mono-infection ($P < 0.05$).

Conclusions： Mutual interaction between *T. gondii* and *E. tenella* were observed in the selected co-infection model. Increased macrophage stress may explain vacuolization and phagocytosis inhibition. In addition to morphologic alterations of macrophages, cytokine up/down- regulation differed between co-infected and mono-infected macrophage cultures. It appears that *E. tenella*, impairs the active penetration of *T. gondii* into host cells which deserves further study. The established *in vitro* infection model may serve to investigate host immune response of macrophages to diverse intracellular pathogens that infect chicken.

6 Zusammenfassung

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***In-vitro*-Koinfektionsstudien mit *Toxoplasma gondii* und *Eimeria tenella* in primären Hühnermakrophagen**

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Schlüsselwörter: *Toxoplasma gondii*, *Eimeria tenella*, Koinfektion, Makrophagen, *in vitro*, Wirt-Parasit-Interaktion, Parasitenwachstum, Wirtsimmunantwort, Cell-imaging

Einleitung: *Toxoplasma gondii* und *Eimeria tenella* sind intrazelluläre apikomplexe Parasiten, die in Hühnern weit verbreitet sind. Während Infektionen mit dem zoonotischen Erreger *T. gondii* beim Geflügel im Allgemeinen subklinisch verlaufen, kann *E. tenella* in Hühnerbeständen schwere Erkrankungen und wirtschaftliche Verluste verursachen. Aufgrund der hohen Seroprävalenz beider Parasiten bei Hühnern sind Koinfektionen wahrscheinlich. Hühnermakrophagen sind wichtig für die Wirtsimmunantwort gegen diese beiden Protozoen. Es ist jedoch wenig über die Wirt-Parasit- sowie Parasit-Parasit-Interaktion in Hühnermakrophagen während einer Koinfektion bekannt.

Ziele der Arbeit: Ein geeignetes In-vitro-Koinfektionsmodell für *T. gondii*- und *E. tenella*-Infektionen in primären Hühnermakrophagen wurde erstellt und angewendet, um die Makrophagenantwort auf beide Parasitenarten und Koinfektionen zu untersuchen.

Tiere, Material und Methoden: Von Monozyten abgeleitete Makrophagen wurden aus Hühnerblut isoliert und aufgereinigt. Eine Koinfektion mit zwei Tachyzoiten des RH-Stammes von *T. gondii* und zwei Sporozoiten des *E. tenella*-Houghton-Stammes pro Zelle wurde gleichzeitig oder nacheinander *in vitro* durchgeführt. Morphologische Veränderungen der Makrophagen und die Parasitenentwicklung wurden mittels Konfokalmikroskopie (CLSM) 2, 6, 12, 24, 48 und 72 Stunden nach Infektion (hpi) visualisiert. Die Parasitenvermehrung wurde evaluiert, indem die Expression des 529-bp-Fragments von *T. gondii* und des ITS-1-Genfragments von *E. tenella* durch qPCR bewertet

wurden. Die Makrophagen-Phagozytose wurde durch Exposition gegenüber pH-sensitiven fluoreszierenden Biopartikeln stimuliert und durch ein dreidimensionales Modell unter Verwendung von CLSM und Imaris®-Software 2, 6, 12 und 24 hpi quantifiziert. Weiterhin wurden während der Infektion relevante Zytokine (IL-6, IL-10, IL-12, iNOS, TNF-α und IFN-γ) durch Genexpressionsanalyse 24, 48 und 72 hpi untersucht. Zusätzlich wurden die Zellinvasion durch und das Überleben von *T. gondii* im Verlauf einer sequentiellen Infektion evaluiert, indem Makrophagen zuvor der Infektion mit *E. tenella* ausgesetzt waren. Die Motilität invasiver Tachyzoiten wurde innerhalb von 20 hpi durch Live-Cell-Imaging verfolgt.

Ergebnisse: Die Makrophagen zeigten eine deutliche immunologische Reaktion und Phagozytoseaktivierung ab 2 hpi. Signifikante Veränderungen der Morphologie mit erhöhter Zellvakuolisierung und -ablösung wurden ab 24 hpi während der Koinfektion beobachtet. Bei zuvor *E. tenella*-exponierten Makrophagen fiel auf, dass *T. gondii* bei hoher Motilität über 4 Stunden an der Makrophagenmembran anhaftete, bevor es zu einer Penetration kam. Ab 24 hpi vermehrten sich *T. gondii* in koinfizierten Makrophagen besser als *E. tenella*. Eine Koinfektion hemmte die Phagozytoseaktivität von Makrophagen nach 2 hpi erheblich, so dass Biopartikel nicht aufgenommen wurden (12 hpi). Mittels qPCR wurde gezeigt, dass bei sequenzieller Koinfektion 2 hpi circa 4-fach weniger *T. gondii* überlebten als bei Monoinfektion ($P < 0,05$). Die Anzahl beider Parasiten nahm während der simultanen Koinfektion zu, aber bei der sequentiellen Koinfektion war die Vermehrung von *T. gondii* im Vergleich zur Monoinfektion bis 24 hpi auf etwa die Hälfte reduziert. Bis 72 hpi verdoppelte sich die Anzahl von *E. tenella*, während *T. gondii* bei Koinfektion in diesem Zeitraum auf dem gleichen Niveau wie 48 hpi blieb. Die mRNA-Expression von iNOS (48 hpi), TNF-α (72 hpi) und von IL-10 (48 hpi und 72 hpi) war während der Koinfektion im Vergleich zur *E. tenella* Monoinfektion signifikant ($P < 0,05$) erhöht.

Schlussfolgerungen: In dem Koinfektionsmodell wurde eine Interaktion zwischen *T. gondii* und *E. tenella* beobachtet. Zusätzlich zu den morphologischen Veränderungen und der Phagozytosehemmung der Makrophagen unterschieden sich die Parasitenvermehrung sowie die Zytokinexpression zwischen Koinfektion und Monoinfektion. *E. tenella* beeinträchtigt das aktive Eindringen von *T. gondii* in Wirtszellen, wie dies erfolgt, ist derzeit noch nicht geklärt.

7 References

Abel G, Szollösi J. Fachet J. Phagocytosis of fluorescent latex microbeads by peritoneal macrophages in different strains of mice: a flow cytometric study. Eur J Immunogenet. 1991;18:239–245.

Allen PC. Nitric oxide production during *Eimeria tenella* infections in chickens. Poult Sci. 1997;76:810–813.

Allen PC. Effects of daily oral doses of L-arginine on coccidiosis infections in chickens. Poult Sci. 1999;78:1506–1509.

Allen PC, Lydon J. Danforth HD. Effects of components of *Artemisia annua* on coccidia infections in chickens. Poult Sci. 1997;76:1156–1163.

Alnassan AA, Shehata AA, Kotsch M, Lendner M, Daugschies A. Bangoura B. Embryonated chicken eggs as an alternative model for mixed *Clostridium perfringens* and *Eimeria tenella* infection in chickens. Parasitol Res. 2013;112:2299–2306.

Alnassan AA, Thabet A, Daugschies A. Bangoura B. *In vitro* efficacy of allicin on chicken *Eimeria tenella* sporozoites. Parasitol Res. 2015;114:3913–3915.

Amer AO. Swanson MS. A phagosome of one's own: a microbial guide to life in the macrophage. Curr Opin Microbiol. 2002;5:56–61.

Andrade RM, Wessendarp M, Gubbels M-J, Striepen B. Subauste CS. CD40 induces macrophage anti–*Toxoplasma gondii* activity by triggering autophagy-dependent fusion of pathogen-containing vacuoles and lysosomes. J Clin Invest. 2006;116:2366–2377.

Araujo MI, Bliss SK, Suzuki Y, Alcaraz A, Denkers EY. Pearce EJ. Interleukin-12 promotes pathologic liver changes and death in mice coinfected with *Schistosoma mansoni* and *Toxoplasma gondii*. Infect Immun. 2001;69:1454–1462.

Ashburn D, Evans R, Chatterton JMW, Joss AWL. Ho-Yen DO. *Toxoplasma* dye test using cell culture derived tachyzoites. J Clin Pathol. 2000;53:630–633.

Bangoura B, Alnassan AA, Lendner M, Shehata AA, Krüger M. Daugschies A. Efficacy of an anticoccidial live vaccine in prevention of necrotic enteritis in chickens. Exp Parasitol.

2014;145:125–134.

Belli SI, Smith NC. Ferguson DJP. The coccidian oocyst: a tough nut to crack! Trends Parasitol. 2006;22:416–423.

Beug H, Kirchbach A von, Döderlein G, Conscience J-F. Graf T. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell. 1979;18:375–390.

Blake DP, Qin Z, Cai J. Smith AL. Development and validation of real-time polymerase chain reaction assays specific to four species of *Eimeria*. Avian Pathol. 2008;37:89–94.

Blake DP. Tomley FM. Securing poultry production from the ever-present *Eimeria* challenge. Trends Parasitol. 2014;30:12–19.

Brooker S, Akhwale W, Pullan R, Estambale B, Clarke SE, Snow RW. Hotez PJ. Epidemiology of plasmodium-helminth co-infection in Africa: populations at risk, potential impact on anemia, and prospects for combining control. Am J Trop Med Hyg. 2007;77:6_Suppl. 88–98.

Butcher BA. Denkers EY. Mechanism of entry determines the ability of *Toxoplasma gondii* to inhibit macrophage proinflammatory cytokine production. Infect Immun. 2002;70:5216–5224.

Byrnes S, Eaton R. Kogut M. *In vitro* interleukin-1 and tumor necrosis factor-alpha production by macrophages from chickens infected with either *Eimeria maxima* or *Eimeria tenella*. Int J Parasitol. 1993;23:639–645.

Challey JR. Burns WC. The invasion of the cecal mucosa by *Eimeria tenella* sporozoites and their transport by macrophages. J Protozool. 1959;6:238–241.

Chang HR, Grau GE. Pechere JC. Role of TNF and IL-1 in infections with *Toxoplasma gondii*. Immunology. 1990;69:33.

Chapman HD. Use of anticoccidial drugs in broiler chickens in the USA: analysis for the years 1995 to 1999. Poult Sci. 2001;80:572–580.

Chapman HD, Barta JR, Blake D, Gruber A, Jenkins M, Smith NC, Suo X. Tomley FM. A selective review of advances in coccidiosis research. Adv Parasitol. 2013;83:93-171.

Chapman HD. Jeffers TK. Vaccination of chickens against coccidiosis ameliorates drug resistance in commercial poultry production. Int J Parasitol Drugs Drug Resist. 2014;4:214–217.

Chapman HD. Shirley MW. The Houghton strain of *Eimeria tenella*. Avian Pathol. 2003;32:115–127.

Chow Y-P, Wan K-L, Blake DP, Tomley F. Nathan S. Immunogenic *Eimeria tenella* glycosylphosphatidylinositol-anchored surface antigens (SAGs) induce inflammatory responses in avian macrophages. PLoS One. 2011;6:e25233.

Christodoulou V, Messaritakis I, Svirinaki E, Tsatsanis C. Antoniou M. *Leishmania infantum* and *Toxoplasma gondii*: Mixed infection of macrophages in vitro and in vivo. Exp Parasitol. 2011;128:279–284.

Clark EL, Tomley FM. Blake DP. Are *Eimeria* genetically diverse, and does it matter? Trends Parasitol. 2017;33:231–241.

Clark JD, Billington K, Bumstead JM, Oakes RD, Soon PE, Sopp P, Tomley FM. Blake DP. A toolbox facilitating stable transfection of *Eimeria* species. Mol Biochem Parasitol. 2008;162:77–86.

Cornelissen JBWJ, Swinkels WJC, Boersma WA. Rebel JMJ. Host response to simultaneous infections with *Eimeria acervulina*, *maxima* and *tenella*. Vet Parasitol. 2009;162:58–66.

Courret N, Darche S, Sonigo P, Milon G, Buzoni-Gâtel D. Tardieux I. CD11c-and CD11b-expressing mouse leukocytes transport single *Toxoplasma gondii* tachyzoites to the brain. Blood. 2006;107:309–316.

Dalloul RA, Bliss TW, Hong Y-H, Ben-Chouikha I, Park DW, Keeler CL. Lillehoj HS. Unique responses of the avian macrophage to different species of *Eimeria*. Mol Immunol. 2007;44:558–566.

Dalloul RA. Lillehoj HS. Poultry coccidiosis. Expert Rev Vaccines. 2006;5:143–163.

David Sibley L. Invasion and intracellular survival by protozoan parasites. Immunol Rev. 2011;240:72–91.

Denkers EY, Kim L. Butcher BA. In the belly of the beast. Cell Microbiol. 2003;5:75–83.

Diab. El-Bahy MM. *Toxoplasma gondii*: virulence of tachyzoites in serum free media at different temperatures. Exp Parasitol. 2008;118:75–79.

Dimier IH, Quéré P, Naciri M. Bout DT. Inhibition of *Eimeria tenella* development *in vitro* mediated by chicken macrophages and fibroblasts treated with chicken cell supernatants with IFN-γ activity. Avian Dis.1998;42:239–247.

Dimier-Poisson IH, Soundouss Z, Naciri M, Bout DT. Quéré P. Mechanisms of the *Eimeria tenella* growth inhibitory activity induced by concanavalin A and reticuloendotheliosis virus supernatants with interferon gamma activity in chicken macrophages and fibroblasts. Avian Dis. 1999;43:65–74.

Dobos-Kovács M, Varga I, Békési L, Drén CN, Németh I. Farkas T. Concurrent cryptosporidiosis and chicken anaemia virus infection in broiler chickens. Avian Pathol. 1994;23:365–368.

Dubey JP. Advances in the life cycle of *Toxoplasma gondii*. Int J Parasitol. 1998;28:1019–1024.

Dubey JP. The scientific basis for prevention of *Toxoplasma gondii* infection: studies on tissue cyst survival, risk factors and hygiene measures. Springer. 2000; 271-275.

Dubey JP. A review of toxoplasmosis in wild birds. Vet Parasitol. 2002;106:121–153.

Dubey JP. *Toxoplasma gondii* infections in chickens (*Gallus domesticus*). Zoonoses Public Health. 2010;57:60–73.

Dubey JP. Beattie CP. Toxoplasmosis of animals and man. CRC Press, Inc. 1988.

Dubey JP, Hill DE, Jones JL, Hightower AW, Kirkland E, Roberts JM, Marcet PL, Lehmann T, Vianna MBC. Miska K. Prevalence of viable *Toxoplasma gondii* in beef, chicken, and pork from retail meat stores in the United States: risk assessment to consumers. J Parasitol. 2005;91:1082–1094.

Dubey JP, Lindsay DS. Speer CA. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev. 1998;11:267–299.

Dubey JP, Salant H, Sreekumar C, Dahl E, Vianna MCB, Shen SK, Kwok OCH, Spira D, Hamburger J. Lehmann TV. High prevalence of *Toxoplasma gondii* in a commercial flock of chickens in Israel, and public health implications of free-range farming. Vet Parasitol. 2004;121:317–322.

Dunay IR, DaMatta RA, Fux B, Presti R, Greco S, Colonna M. Sibley LD. Gr1+ inflammatory monocytes are required for mucosal resistance to the pathogen *Toxoplasma gondii*. Immunity. 2008;29:306–317.

Ekert J, Braun R, Shirley MW. Courdet P. Biotechnology Guidelines on Techniques in Coccidiosis Research. European Coorperation in the field of and Technical Research. COST. 1992;89:220–221.

Evans R, Chatterton JMW, Ashburn D, Joss AWL. Ho-Yen DO. Cell-culture system for continuous production of *Toxoplasma gondii* tachyzoites. Eur J Clin Microbiol Infect Dis. 1999;18:879–884.

Felger I, Smith T, Edoh D, Kitua A, Alonso P, Tanner M. Beck H-P. 6. multiple *Plasmodium falciparum* infections in Tanzanian infants. Trans R Soc Trop Med Hyg. 1999;93:29–34.

Fichera ME, Bhopale MK. Roos DS. *In vitro* assays elucidate peculiar kinetics of clindamycin action against *Toxoplasma gondii*. Antimicrob Agents Chemother. 1995;39:1530–1537.

Fischer H-G, Nitzgen B, Reichmann G, Groß U. Hadding U. Host cells of *Toxoplasma gondii* encystation in infected primary culture from mouse brain. Parasitol Res. 1997;83:637–641.

Fletcher OJ, Munnell JF. Page RK. Cryptosporidiosis of the bursa of Fabricius of chickens. Avian Dis.1975.630–639.

Frontera E, Alcaide M, Dominguez-Alpizar JL, Boes J, Reina D. Navarrete I. Evidence of interaction between *Ascaris suum* and *Metastrongylus apri* in experimentally infected pigs. Vet Parasitol. 2005;127:295–301.

Gal A, Harrus S, Arcoh I, Lavy E, Aizenberg I, Mekuzas-Yisaschar Y. Baneth G. Coinfection with multiple tick-borne and intestinal parasites in a 6-week-old dog. Can Vet J. 2007;48:619.

Garrido D, Alber A, Kut E, Chanteloup NK, Lion A, Trotereau A, Dupont J, Tedin K, Kaspers B. Vervelde L. The role of type I interferons (IFNs) in the regulation of chicken macrophage inflammatory response to bacterial challenge. Dev Comp Immunol. 2018;86:156–170.

Gazzinelli RT, Denkers EY. Sher A. Host resistance to *Toxoplasma gondii*: model for studying the selective induction of cell-mediated immunity by intracellular parasites. Infect Agents Dis. 1993a;2:139–149.

Gazzinelli RT, Eltoum I, Wynn TA. Sher A. Acute cerebral toxoplasmosis is induced by *in vivo* neutralization of TNF-alpha and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. J Immunol. 1993b;151:3672–3681.

Gazzinelli RT, Oswald IP, James SL. Sher A. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages. J Immunol. 1992;148:1792–1796.

Gazzinelli RT, Wysocka M, Hieny S, Scharton-Kersten T, Cheever A, Kühn R, Müller W, Trinchieri G. Sher A. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol. 1996;157:798–805.

Gessani S. Belardelli F. IFN-γ expression in macrophages and its possible biological significance.

Cytokine Growth Factor Rev. 1998;9:117–123.

Goodwin MA, Dubey JP. Hatkin J. *Toxoplasma gondii* peripheral neuritis in chickens. J Vet Diagn Invest. 1994;6:382–385.

Goren MB. Phagocyte lysosomes: interactions with infectious agents, phagosomes, and experimental perturbations in function. Annu Rev Microbiol. 1977;31:507–533.

Gubbels M-J, Wieffer M. Striepen B. Fluorescent protein tagging in *Toxoplasma gondii*: identification of a novel inner membrane complex component conserved among *Apicomplexa*. Mol Biochem Parasitol. 2004;137:99–110.

Guerrero OM, Chinchilla M. Abrahams E. Increasing of *Toxoplasma gondii* (Coccidia, Sarcocystidae) infections by *Trypanosoma lewisi* (Kinetoplastida, Trypanosomatidae) in white rats. Rev Biol Trop. 1997;45:877–882.

Guillermo L. DaMatta R. Nitric oxide inhibition after *Toxoplasma gondii* infection of chicken macrophage cell lines. Poult Sci. 2004a;83:776–782.

Guy JS, Levy MG, Ley DH, Barnes HJ. Gerig TM. Interaction of reovirus and *Cryptosporidium baileyi* in experimentally infected chickens. Avian Dis. 1988;381–390.

He H, Genovese KJ. Kogut MH. Modulation of chicken macrophage effector function by TH 1/TH 2 cytokines. Cytokine. 2011;53:363–369.

Hepding L. Ueber Toxoplasmen (*Toxoplasma gallinarum* n. sp.) in der Retina eines Huhnes und über deren Beziehung zur Hühnerlähmung. Z. f. Infektionskrankheiten der Haustiere. 1939;55:109–116.

Hériveau C, Dimier-Poisson I, Lowenthal J, Naciri M. Quéré P. Inhibition of *Eimeria tenella* replication after recombinant IFN-γ activation in chicken macrophages, fibroblasts and epithelial cells. Vet Parasitol. 2000;92:37–49.

Hibbs JB, Taintor RR. Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science. 1987;235:473–476.

Hiob L, Koethe M, Schares G, Goroll T, Daugschies A. Bangoura B. Experimental *Toxoplasma gondii* and *Eimeria tenella* co-infection in chickens. Parasitol Res. 2017;116:3189-3203.

Hofmann J. Raether W. Improved techniques for the *in vitro* cultivation of *Eimeria tenella* in primary chick kidney cells. Parasitol Res. 1990;76:479–486.

Hong YH, Lillehoj HS, Lee SH, Dalloul RA. Lillehoj EP. Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. Vet Immunol Immunopathol. 2006;114:209–223.

Hughes HPA. Fleck DG. *In vitro* culture of *Toxoplasma gondii* in primary and established cell lines. Int J Parasitol. 1986;16:317–322.

Inoue M, Yamamoto H. Hihara H. Fc receptor expression and immune phagocytosis in chicken monocytic cell lines. J Vet Med Sci. 1992;54:591–594.

Jackson MH. Hutchison WM. The prevalence and source of *Toxoplasma* infection in the environment. Academic Press. 1989:28:55-105.

Jahiel RI, Vilcek J, Nussenzweig R. Vanderberg J. Interferon inducers protect mice against *Plasmodium berghei* malaria. Science. 1968;161:802–804.

Jenkins M, Allen P, Wilkins G, Klopp S. Miska K. *Eimeria praecox* infection ameliorates effects of *Eimeria maxima* infection in chickens. Vet Parasitol. 2008;155:10–14.

Jenkins M, Fetterer R. Miska K. Co-infection of chickens with *Eimeria praecox* and *Eimeria maxima* does not prevent development of immunity to *Eimeria maxima*. Vet Parasitol. 2009;161:320–323.

Jeurissen SH. Janse EM. Distribution and function of non-lymphoid cells in liver and spleen of embryonic and adult chickens. Prog Clin Biol Res. 1989;307:149-157.

Jeurissen SHM, Janse EM, Vermeulen AN. Vervelde L. *Eimeria tenella* infections in chickens: aspects of host-parasite: interaction. Vet Immunol Immunopathol. 1996;54:231–238.

Jones JL. Dubey JP. Foodborne toxoplasmosis. Clin Infect Dis. 2012;55:845–851.

Juul-Madsen HR, Viertlboeck B, Smith AL. Göbel TWF. Avian innate immune responses. Avian immunology. 2008.129–158.

Kapellos TS, Taylor L, Lee H, Cowley SA, James WS, Iqbal AJ. Greaves DR. A novel real time imaging platform to quantify macrophage phagocytosis. Biochem Pharmacol. 2016;116:107–119.

Kawahara F, Taira K, Nagai S, Onaga H, Onuma M. Nunoya T. Detection of five avian *Eimeria* species by species-specific real-time polymerase chain reaction assay. Avian Dis. 2008;52:652–656.

Khalafalla RE, Muller U, Shahiduzzaman M, Dyachenko V, Desouky AY, Alber G. Daugschies A.

Effects of curcumin (diferuloylmethane) on *Eimeria tenella* sporozoites in vitro. Parasitol Res. 2011;108:879–886.

Kodukula P, Liu T, van Rooijen N, Jager MJ. Hendricks RL. Macrophage control of herpes simplex virus type 1 replication in the peripheral nervous system. J Immunol. 1999;162:2895–2905.

Koethe M, Pott S, Ludewig M, Bangoura B, Zoller B, Daugschies A, Tenter AM, Spekker K, Bittame A, Mercier C, Fehlhaber K. Straubinger RK. Prevalence of specific IgG-antibodies against *Toxoplasma gondii* in domestic turkeys determined by kinetic ELISA based on recombinant GRA7 and GRA8. Vet Parasitol. 2011;180:179–190.

Kvičerová J, Pakandl M. Hypša V. Phylogenetic relationships among *Eimeria* spp.(*Apicomplexa*, *Eimeriidae*) infecting rabbits. Parasitology. 2008;135:443–452.

Lal K, Bromley E, Oakes R, Prieto JH, Sanderson SJ, Kurian D, Hunt L, Yates III JR, Wastling JM. Sinden RE. Proteomic comparison of four *Eimeria tenella* life-cycle stages: Unsporulated oocyst, sporulated oocyst, sporozoite and second-generation merozoite. Proteomics. 2009;9:4566–4576.

Laurent F, Mancassola R, Lacroix S, Menezes R. Naciri M. Analysis of chicken mucosal immune response to *Eimeria tenella* and *Eimeria maxima* infection by quantitative reverse transcription-PCR. Infect Immun. 2001;69:2527–2534.

Lemaitre B. The road to Toll. Nat Rev Immunol. 2004;4:521.

Letscher-Bru V, Villard O, Risse B, Zauke M, Klein J-P. Kien TT. Protective effect of vaccination with a combination of recombinant surface antigen 1 and interleukin-12 against toxoplasmosis in mice. Infect Immun. 1998;66:4503–4506.

Lieberman LA. Hunter CA. The role of cytokines and their signaling pathways in the regulation of immunity to *Toxoplasma gondii*. Int Rev Immunol. 2009;21:373–403.

Lillehoj HS. Role of T lymphocytes and cytokines in coccidiosis. Int J Parasitol. 1998;28:1071–1081.

Lillehoj HS. Choi KD. Recombinant chicken interferon-gamma-mediated inhibition of *Eimeria tenella* development *in vitro* and reduction of oocyst production and body weight loss following *Eimeria acervulina* challenge infection. Avian Dis. 1998;42:307–314.

Lillehoj HS, Dalloul RA. Min W. Enhancing intestinal immunity to coccidiosis. World Poult. 2003;19:18–21.

Lillehoj HS. Li G. Nitric oxide production by macrophages stimulated with coccidia sporozoites, lipopolysaccharide, or interferon-γ, and its dynamic changes in SC and TK strains of chickens infected with *Eimeria tenella*. Avian Dis. 2004;48:244–253.

Lillehoj HS, Min W. Dalloul RA. Recent progress on the cytokine regulation of intestinal immune responses to *Eimeria*. Poult Sci. 2004;83:611–623.

Ling YM, Shaw MH, Ayala C, Coppens I, Taylor GA, Ferguson DJP. Yap GS. Vacuolar and plasma membrane stripping and autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. J Exp Med. 2006;203:2063–2071.

Long PL. Pierce AE. Role of cellular factors in the mediation of immunity to avian coccidiosis (*Eimeria tenella*). Nature. 1963;200:426-427.

Long PL. Rose ME. Growth of *Eimeria tenella* in vitro in macrophages from chicken peritoneal exudates. Z. Parasitenk. 1976;48:291–294.

Ludlow LE, Zhou J, Tippett E, Cheng W-J, Hasang W, Rogerson SJ. Jaworowski A. HIV-1 inhibits phagocytosis and inflammatory cytokine responses of human monocyte-derived macrophages to *P. falciparum* infected erythrocytes. PLoS One. 2012;7:e32102.

Lyons RE, Anthony JP, Ferguson DJP, Byrne N, Alexander J, Roberts F. Roberts CW. Immunological studies of chronic ocular toxoplasmosis. Infect Immun. 2001;69:2589–2595.

Maksimov P, Buschtöns S, Herrmann DC, Conraths FJ, Görlich K, Am Tenter, Dubey JP, Nagel-Kohl U, Thoms B. Böttcher L. Serological survey and risk factors for *Toxoplasma gondii* in domestic ducks and geese in Lower Saxony, Germany. Vet Parasitol. 2011;182:140–149.

Malkwitz I, Berndt A, Daugschies A. Bangoura B. Long-term investigations on *Toxoplasma gondii*-infected primary chicken macrophages. Parasitol Res. 2013;112:3115–3122.

Malkwitz I, Berndt A, Daugschies A. Bangoura B. Characterisation of susceptibility of chicken macrophages to infection with *Toxoplasma gondii* of type II and III strains. Exp Parasitol. 2018;187:22-29.

Manwell RD, Coulston F, Binckley EC. Jones VP. Mammalian and avian *Toxoplasma*. J Infect Dis. 1945;76:1–14.

Marshall AJ, Brunet LR, van Gessel Y, Alcaraz A, Bliss SK, Pearce EJ. Denkers EY. *Toxoplasma gondii* and *Schistosoma mansoni* synergize to promote hepatocyte dysfunction associated with high

levels of plasma TNF-α and early death in C57BL/6 mice. J Immunol. 1999;163:2089–2097.

Mason S, Dubey JP, Smith JE. Boag B. *Toxoplasma gondii* coinfection with diseases and parasites in wild rabbits in Scotland. Parasitology. 2015;142:1415–1421.

Meirelles MN. De WS. Killing of *Trypanosoma cruzi* and *Leishmania mexicana*, and survival of *Toxoplasma gondii*, in chicken macrophages *in vitro*. Journal of submicroscopic cytology. 1985;17:327–334.

Meirelles MN. Souza W de. Killing of Trypanosoma cruzi and Leishmania mexicana, and survival of *Toxoplasma gondii*, in chicken macrophages in vitro. J Submicrosc Cytol. 1985;17:3.327–334.

Milanova A, Santos R, Lashev L, Koinarski V. Fink-Gremmels J. Influence of experimentally induced *Eimeria tenella* infection on gene expression of some host response factors in chickens. Bulg J Vet Med. 2016;19:47–56.

Mordue DG, Håkansson S, Niesman I. Sibley LD. *Toxoplasma gondii* resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways. Exp Parasitol. 1999;92:87–99.

Mordue DG. Sibley LD. Intracellular fate of vacuoles containing *Toxoplasma gondii* is determined at the time of formation and depends on the mechanism of entry. J Immunol. 1997;159:4452–4459.

Morisaki JH, Heuser JE. Sibley LD. Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. J Cell Sci. 1995;108:2457–2464.

Moura MdA, Amendoeira MRR. Barbosa HS. Primary culture of intestinal epithelial cells as a potential model for *Toxoplasma gondii* enteric cycle studies. Mem Inst Oswaldo Cruz. 2009;104:862–864.

Muller J. Hemphill A. *In vitro* culture systems for the study of apicomplexan parasites in farm animals. Int J Parasitol. 2013;43:115–124.

Murray HW. Nathan CF. Macrophage microbicidal mechanisms *in vivo*: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. J Exp Med. 1999;189:741–746.

Nagineni CN, Pardhasaradhi K, Martins MC, Detrick B. Hooks JJ. Mechanisms of interferon-induced inhibition of *Toxoplasma gondii* replication in human retinal pigment epithelial cells. Infect Immun. 1996;64:4188–4196.

Niikura M, Kamiya S, Kita K. Kobayashi F. Coinfection with nonlethal murine malaria parasites suppresses pathogenesis caused by *Plasmodium berghei* NK65. J Immunol. 2008;180:6877–6884.

Ogedengbe JD, Hanner RH. Barta JR. DNA barcoding identifies *Eimeria* species and contributes to the phylogenetics of coccidian parasites (*Eimeriorina*, *Apicomplexa*, *Alveolata*). Int J Parasitol. 2011a;41:843–850.

Ogedengbe JD, Hunter DB. Barta JR. Molecular identification of *Eimeria* species infecting market-age meat chickens in commercial flocks in Ontario. Vet Parasitol. 2011b;178:350–354.

Okamura M, Lillehoj HS, Raybourne RB, Babu US, Heckert RA, Tani H, Sasai K, Baba E. Lillehoj EP. Differential responses of macrophages to *Salmonella enterica* serovars *Enteritidis* and *Typhimurium*. Vet Immunol Immunopathol. 2005;107:327–335.

Onaga H, Tajima M. Ishii T. Activation of macrophages by culture fluid of antigen-stimulated spleen cells collected from chickens immunized with *Eimeria tenella*. Vet Parasitol. 1983;13:1–11.

Otranto D, Testini G, Buonavoglia C, Parisi A, Brandonisio O, Circella E, Dantas-Torres F. Camarda A. Experimental and field investigations on the role of birds as hosts of *Leishmania infantum*, with emphasis on the domestic chicken. Acta tropica. 2010;113:80–83.

Patton WH. *Eimeria tenella*: cultivation of the asexual stages in cultured animal cells. Science. 1965;150:767–769.

Peek HW. Landman WJM. Higher incidence of *Eimeria* spp. field isolates sensitive for diclazuril and monensin associated with the use of live coccidiosis vaccination with Paracox™-5 in broiler farms. Avian Dis. 2006;50:434–439.

Philip R. Epstein LB. Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon and interleukin-1. Nature. 1986;323:86-89.

Quéré P, Pierre J, Hoang M-D, Esnault E, Domenech J, Sibille P. Dimier-Poisson I. Presence of dendritic cells in chicken spleen cell preparations and their functional interaction with the parasite *Toxoplasma gondii*. Vet Immunol Immunopathol. 2013;153:57–69.

Qureshi MA. Avian macrophage and immune response: an overview. Poult Sci. 2003;82:691–698.

Qureshi MA. Dietert RR. Bacterial uptake and killing by macrophages. Methods Immunotoxicol. 1995;119–131.

Qureshi MA, Heggen CL. Hussain I. Avian macrophage: effector functions in health and disease. Dev Comp Immunol. 2000;24:103–119.

Qureshi MA, Miller L, Lillehoj HS. Ficken MD. Establishment and characterization of a chicken mononuclear cell line. Vet Immunol Immunopathol. 1990;26:237–250.

Qureshi MA, Petitte JN, Laster SM. Dietert RR. Avian macrophages: contribution to cellular microenvironment and changes in effector functions following activation. Poult Sci. 1993;72:1280–1284.

Rathinam T. Chapman HD. Sensitivity of isolates of *Eimeria* from turkey flocks to the anticoccidial drugs amprolium, clopidol, diclazuril, and monensin. Avian Dis. 2009;53:405–408.

Rifaat MA, Salem SA, Azab ME, el-Razik IA, Safer EH, Beshir, SR. el-Shennawy SF. Experimental concomitant toxoplasma and malaria infection in rats. Folia Parasitol. 1984;31:97–104.

Ritter GD, Ley DH, Levy M, Guy J. Barnes HJ. Intestinal cryptosporidiosis and reovirus isolation from bobwhite quail (*Colinus virginianus*) with enteritis. Avian Dis. 1986;603–608.

Robben PM, Mordue DG, Truscott SM, Takeda K, Akira S. Sibley LD. Production of IL-12 by macrophages infected with *Toxoplasma gondii* depends on the parasite genotype. J Immunol. 2004;172:6.3686–3694.

Robert-Gangneux F. Dardé M-L. Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev. 2012;25:264–296.

Romano JD, Beaumont C de, Carrasco JA, Ehrenman K, Bavoil PM. Coppens I. Fierce competition between *Toxoplasma* and *Chlamydia* for host cell structures in dually infected cells. Eukaryot Cell. 2013;12:265–277.

Rose ME. Lee DL. Interactions in vitro between sporozoites of *Eimeria tenella* and host peritoneal exudate cells: electron microscopal observations. Z. Parasitenk. 1977;54:1–7.

Rossi G. Turba F. Phagocytosis of differently treated Candida cells by chicken bone marrow-and peritoneal macrophages (author's transl). Mykosen. 1981;24:684–694.

Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, Smith AL. Kaiser P. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J Immunol. 2004;173:2675–2682.

Ruff MD. Important parasites in poultry production systems. Vet Parasitol. 1999;84:337–347.

Ryning FW. Remington JS. Effect of cytochalasin D on *Toxoplasma gondii* cell entry. Infect Immun. 1978;20:739–743.

Saeij JPJ, Boyle JP. Boothroyd JC. Differences among the three major strains of *Toxoplasma gondii* and their specific interactions with the infected host. Trends Parasitol. 2005;21:476–481.

Sasai M, Pradipta A. Yamamoto M. Host immune responses to *Toxoplasma gondii*. Int Immunol. 2018;30:113–119.

Scharton-Kersten TM, Yap G, Magram J. Sher A. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen *Toxoplasma gondii*. J Exp Med. 1997;185:1261–1274.

Sharma J. Avian cellular immunology. Routledge. 2018.

Shi TY, Liu XY, Hao LL, Li JD, Gh AN, Abdille MH. Suo X. Transfected *Eimeria tenella* could complete its endogenous development *in vitro*. J Parasitol. 2008;94:978–981.

Shirley MW. Lillehoj HS. The long view: a selective review of 40 years of coccidiosis research. Avian Pathol. 2012;41:111–121.

Shivaramaiah C, Barta, JR, Hernandez-Velasco X, Téllez G. Hargis B. Coccidiosis. Vet Med. 2014;5:23–34.

Sibley LD, Weidner E. Krahenbuhl JL. Phagosome acidification blocked by intracellular *Toxoplasma gondii*. Nature. 1985;315:416-419.

Skariah S, McIntyre MK. Mordue DG. *Toxoplasma gondii*: determinants of tachyzoite to bradyzoite conversion. Parasitol Res. 2010;107:253–260.

Smith II CK. Strout RG. *Eimeria tenella*: accumulation and retention of anticoccidial ionophores by extracellular sporozoites. Exp Parasitol. 1979;48:325–330.

Soares Medeiros LC, Gomes F, Maciel LR, Seabra SH, Docampo R, Moreno S, Plattner H, Hentschel J, Kawazoe U. Barrabin H. Volutin granules of *Eimeria* parasites are acidic compartments and have physiological and structural characteristics similar to acidocalcisomes. J Protozool. 2011;58:416–423.

Sundermann CA. Estridge BH. Growth of and competition between *Neospora caninum* and *Toxoplasma gondii in vitro*. Int J Parasitol. 1999;29:1725–1732.

Suzuki Y, Orellana MA, Schreiber RD. Remington JS. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. Science. 1988;240:516–518.

Takács AC, Swierzy IJ. Lüder CGK. Interferon-γ restricts *Toxoplasma gondii* development in murine skeletal muscle cells via nitric oxide production and immunity-related GTPases. PLoS One. 2012;7:e45440.

Tang X, Yin G, Qin M, Tao G, Suo J, Liu X. Suo X. Transgenic *Eimeria tenella* as a vaccine vehicle. Sci Rep. 2016;6:29379.

Taubert A, Behrendt JH, Sühwold A, Zahner H. Hermosilla C. Monocyte-and macrophage-mediated immune reactions against *Eimeria bovis*. Vet Parasitol. 2009;164:141–153.

Telfer S, Birtles R, Bennett M, Lambin X, Paterson S. Begon M. Parasite interactions in natural populations: insights from longitudinal data. Parasitology. 2008;135:767–781.

Tenter AM, Heckeroth AR. Weiss LM. *Toxoplasma gondii*. Int J Parasitol. 2000;30:1217–1258.

Thabet A, Zhang R, Alnassan A-A, Daugschies A. Bangoura B. Anticoccidial efficacy testing: *In vitro Eimeria tenella* assays as replacement for animal experiments. Vet Parasitol. 2017;233:86–96.

Tierney J, Gowing H, van Sinderen D, Flynn S, Stanley L, McHardy N, Hallahan S. Mulcahy G. *In vitro* inhibition of *Eimeria tenella* invasion by indigenous chicken *Lactobacillus* species. Vet Parasitol. 2004;122:171–182.

Tierney J. Mulcahy G. Comparative development of *Eimeria tenella* (*Apicomplexa*) in host cells in vitro. Parasitol Res. 2003;90:301–304.

Torgerson PR. Mastroiacovo P. The global burden of congenital toxoplasmosis: a systematic review. Bull World Health Organ. 2013;91:501–508.

Tosh KW, Mittereder L, Bonne-Annee S, Hieny S, Nutman TB, Singer SM, Sher A. Jankovic D. The IL-12 response of primary human dendritic cells and monocytes to *Toxoplasma gondii* is stimulated by phagocytosis of live parasites rather than host cell invasion. J Immunol. 2016;196:345–356.

Trout JM. Lillehoi HS. Evidence of a role for intestinal CD8+ lymphocytes and macrophages in transport of *Eimeria acervulina* sporozoites. J Parasitol. 1993;79:790–792.

Unno A, Suzuki K, Xuan X, Nishikawa Y, Kitoh K. Takashima Y. Dissemination of extracellular and intracellular *Toxoplasma gondii* tachyzoites in the blood flow. Parasitol Int. 2008;57:515–518.

Vervelde L, Vermeulen AN. Jeurissen SHM. *In situ* characterization of leucocyte subpopulations after infection with *Eimeria tenella* in chickens. Parasite Immunol. 1996;18:247–256.

Vischer WA. Suter E. Intracellular multiplication of *Toxoplasma gondii* in adult mammalian macrophages cultivated *in vitro*. Proc Soc Exp Biol Med. 1954;86:413–419.

Wille U, Villegas EN, Striepen B, Roos DS. Hunter CA. Interleukin-10 does not contribute to the pathogenesis of a virulent strain of *Toxoplasma gondii*. Parasite Immunol. 2001;23:291–296.

Xing Z, Cardona CJ, Anunciacion J, Adams S. Dao N. Roles of the ERK MAPK in the regulation of proinflammatory and apoptotic responses in chicken macrophages infected with H9N2 avian influenza virus. J Gen Virol. 2010;91:343–351.

Yang H, Zhang A, Yang Y. Qian Z. Study on invasibility and multiplication of *Toxoplasma* strains with different virulence toward Vero-cells. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 1998;16:185–188.

Yilmaz A, Shen S, Adelson DL, Xavier S. Zhu JJ. Identification and sequence analysis of chicken Toll-like receptors. Immunogenetics. 2005;56:743–753.

Yoeli M, Becker Y. Bernkopf H. The effect of west nile virus on experimental malaria infection (*Plasmodium betrghei*) in mice. Harefuah. 1955;49:6.

Youn HJ. Noh JW. Screening of the anticoccidial effects of herb extracts against *Eimeria tenella*. Vet Parasitol. 2001;96:257–263.

Yun CH, Lillehoj HS. Choi KD. *Eimeria tenella* infection induces local gamma interferon production and intestinal lymphocyte subpopulation changes. Infect Immun. 2000;68:1282–1288.

Yurayart N, Kaewthamasorn M. Tiawsirisup S. Vector competence of *Aedes albopictus* (Skuse) and *Aedes aegypti* (Linnaeus) for *Plasmodium gallinaceum* infection and transmission. Vet Parasitol. 2017;241:20–25.

Zhang J, Wilson E, Yang S. Healey MC. Adapting *Eimeria tenella* to grow in primary chicken kidney cells following repeated passages between cell culture and chickens. Avian Dis. 1997;41:111–116.

Zhang S, Lillehoj HS. Ruff MD. Chicken Tumor Necrosis-Like Factor. 1. *In vitro* production by

macrophages stimulated with *Eimeria tenella* or bacterial lipopolysaccharide. Poult Sci. 1995a; 74:1304–1310.

Zhang S, Lillehoj HS. Ruff MD. In vivo role of tumor necrosis-like factor in *Eimeria tenella* infection. Avian Dis. 1995b;859–866.

Zhao Y, Ferguson DJP, Wilson DC, Howard JC, Sibley LD. Yap GS. Virulent *Toxoplasma gondii* evade immunity-related GTPase-mediated parasite vacuole disruption within primed macrophages. J Immunol. 2009;182:3775–3781.

Zou J, Huang X-X, Yin G-W, Ding Y, Liu X-Y, Wang H, Chen Q-J. Suo X. Evaluation of *Toxoplasma gondii* as a live vaccine vector in susceptible and resistant hosts. Parasit Vectors. 2011;4:168.

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